

nature*December 25, 1975*

New era of firmer control in French science

SUPPORT for science marks time. Universities live on budgets which in some countries, the UK included, have recently shrunk in real terms. Government interest in science is, at best, lukewarm. Students decide in large numbers that a career in science is not for them. Everywhere the scientist finds himself less loved than he used to be. Almost everywhere, that is; some interesting things are happening in France.

During the Gaullist period, French science grew at a healthy rate—in 1967, for example, research and development accounted for about 2.1% of the GNP. In subsequent years this figure declined; by 1974 it was down to 1.7%. Now President Giscard d'Estaing seems to have made a positive decision, after an eighteen-month policy review, to go for growth again. It is likely that, having seen that some other comparable industrial countries are spending rather more than 2% of their GNP on research and development, he will attempt to bring France up to that level again. A recent council at the Elysée Palace—the second in a year—wrestled with the problem of how to bring about the growth.

The President is not ordering this expansion for the sake of increasing prestige. He sees that by the year 2000 France's manpower resources will not have risen substantially, and so it will be increasingly relying on the export of technological expertise to keep its place among the rich nations. It is largely for the maintenance of France's intellectual resources to do just this that Giscard d'Estaing wishes to increase his support for science. The new policy can be seen to have two distinct aspects: reorganisation of research priorities, and the creation of more employment opportunities.

In the past, various organisations have sprung up to carry out specific tasks; they have included IRIA (for Information Processing and Automation), CNES (for space research), CNEXO (for ocean exploration) and ORSTOM (for overseas research). As time progressed the missions of these bodies changed somewhat in

response to both internal and external pressures. But they had no monopoly on research in their own field, and friction developed as questions of 'who does what' arose. Now it is proposed that consultative committees be established to act as buffers between the individual organisations and the Minister for Industry and Research, M d'Ornano. These committees will, it is expected, make it more difficult for scientists to act both as appellant and judge in deciding what projects should be undertaken. M d'Ornano has made no secret of his wish to have firmer control over the work done in the laboratories, and this new mechanism will undoubtedly help him, although it may not be well received by the research workers' unions, who have come to expect increased participation of workers in decision-making on matters of policy.

On the other hand, the unions will probably be fairly satisfied by improvements being made in terms of employment. In recent years a growing number of French scientists have found themselves having to work on short-term contracts, as tenured posts have dried up. This problem is now to be tackled; many thousand posts for researchers and technicians which at present carry little security are to be converted, in the next five years, into more stable positions. And, as an incentive to young students to work in fields which the government judges to be of priority, up to 1,500 studentships will be created, paying 2,000 francs per month for the second and third years of postgraduate thesis work.

There are risks in this, of course. Fundamental research could well suffer if the mood that research should satisfy national goals becomes too strong. It would be difficult to demonstrate that the UK had, in the post-Rothschild period, lost out on fundamental research while attempting to stimulate more applied research. But the universities of France do not have as strong a tradition as do the British ones of acting as the guardians of fundamental research.



A justifiable case for support?

The passing of the old year next week will be celebrated with no little fervour by the UK computer industry. The economic recession triggered by the oil crisis really hit hard during 1975, so much so that survival rather than growth became the priority, and the short-term objectives of maintaining profitability and, even more important, good cash-flow, tended to override all other considerations. Nicholas Enticknap reviews the state of the industry.

MOST British computer companies can look back on the year with satisfaction. The most important of them, International Computers Ltd (ICL), has just reported its trading figures for the financial year ending September 30, and these show a 20% increase in turnover, less than half of which is accounted for by price rises, and a 36% increase in profit after tax. Another leading company, Data Recording Instrument Co., continued to increase its exports and was able to set up a new subsidiary in France. Two of Britain's largest computer service companies, CMG Computer Management Group and Logica, both increased their turnover by more than 50% though pre-tax profits were less than hoped for.

On the debit side, Core-Computer Related Equipment, a distributor of imported computer equipment, went into liquidation last month. And a little earlier, Process Peripherals, a company which specialises in specially strengthened data storage devices, ran out of money, in spite of a large and healthy order book. The company's assets have, however, been bought by a British electronic company, Gresham-Lion, and this has enabled Process Peripherals to carry on.

Looking at the UK industry in a broader context, though, it is still nowhere near to supplying the needs of the country's users, far less becoming a net exporter. The most recent year's figures supplied by the Department of Industry are for 1974; they show exports of £210 million and imports of £346 million, a net deficit of £136 million. Total sales of the industry were £517 million. The figures for the first six months of 1975 are similarly depressing.

But these figures, bad as they are, actually flatter British industry, for they include sales of equipment manufactured in Britain by foreign manufacturers. Four of the top five American computer companies, IBM, Honeywell, Burroughs, and NCR, have factories in the UK, most of them located in Scotland. These factories do, of course, contribute to the balance of payments and provide employment, but the profits end up in American rather

than British pockets.

Market shares provide another measure of the success of British industry, though admittedly a controversial one as the arguments over the definition of the market and the best method of valuation are endless. According to ICL executives, who can reasonably be expected to be as favourable to ICL as anybody, their company is maintaining a steady 38% share of the total value of all computers installed in the UK. No other British company has a significant share, so well over half the computer equipment in use in Britain was made by foreign companies.

This again, unsatisfactory though it is, is flattering rather than the reverse, because, for example, many of ICL's computers use disk drives designed and manufactured in America, and these can account for a substantial proportion of a computer system's total value. Nonetheless ICL, which was formed by merger of several different computer companies in 1968, has done well; it has a larger share of its home market than any major computer company, other than IBM, albeit in somewhat artificial market conditions. IBM is the giant of the industry, and has sold between 60 and 70% of the total value of all computers installed worldwide—only in Britain and Japan of the industrialised countries does it have less than 50%.

ICL sells 'mainframe' computers, which are general purpose systems ranging in value from around £30,000 to several millions. Another class of computer, the minicomputer, is normally used for one or two specific tasks such as control of an industrial process, but is increasingly being adopted for commercial use. Prices range from around £1,000 up to around £100,000, but nonetheless, minicomputer business is big business—the leading manufacturer, Digital Equipment Corp., has a turnover of \$500 million.

Britain's record in this area is little short of appalling. There has been no attempt to merge the number of small companies competing with each other into one cohesive unit, with the result that none of them has become a major force, and most of them have suffered financial disaster. Ferranti, whose

troubles are well known, is the biggest producer, with a manufacturing volume of around 30 systems a month. This compares with over 100 produced by the Swedish company Datasaab, and volumes ranging from 350 to 500 for the leading American manufacturers.

This record is not due to poor design, for British minicomputers have generally received high marks for technological expertise and innovation in independent surveys. It is primarily due to the limited size of the home market, which simply cannot support a large number of companies. This means that development costs are spread over a smaller number of units, so prices are higher and contracts are lost. Furthermore, there is no preferential purchasing policy by government departments to compare with that enjoyed by ICL.

The question of government involvement in the computer industry is a perennial source of concern. There can be no doubt that the government should formulate a policy. The net deficit in the balance of payments should in itself be a matter of national concern, the more so as sales of computer equipment are projected to grow by 15% a year in the UK into the indefinite future. The types of computer equipment that have the biggest growth potential—minicomputers, terminals and microcomputers—are all dominated by American companies to an even greater extent than the mainframe business, so the trading imbalance is likely to get much worse unless corrective action is taken.

In spite of this, the government has never had a planned policy for the computer industry, and has merely responded to individual problems when sufficient pressure has been exerted. This is extraordinary considering the enormous sums of money spent on the motor industry this year in the face of advice from government bodies that there is little prospect of a return on this investment. The bills for Concorde have come to around £1,500 million, spent in the cause of developing new technology. Yet the computer industry both embodies new technology and offers hope for a return on investment, but has only received government funds to the tune of around £50 million since the formation of ICL.

This £50 million is largely accounted for by an investment-free loan of £40 million to ICL for development of its latest range of computers, the 2900 series. Other smaller sums have been made available to a number of companies through the National Research Development Corporation and the National Computing Centre, but these have all funded a maximum of half the total cost of each development project. The government does help in one

other way, by requiring its own departments to buy British (which means ICL) when purchasing any computer with more than a specified amount of computer power.

One result of this lack of support is to induce British companies to adopt an "if you can't beat them, join them" policy. ICL has joined in a three-sided agreement with two American companies, NCR and Control Data Corporation, covering the development and manufacturer of peripherals. Data

Recording Instrument Company has similarly made agreements with various North American companies concerning the supply and manufacture of disk drives.

It is depressing to move into 1976 without any sign of a change of attitude from the British government. Certainly none could be detected in the speech made by Eric Varley, Secretary of State for Industry, at the opening this month of the National Computing Centre's new headquarters. Still, it

could be worse. One of the saddest events of 1975 was the collapse of Unidata, a pan-European computer manufacturer formed from the French government company *Compagnie Internationale pour l'Informatique*, and from the computer subsidiaries of Philips of Holland and Siemens of Germany. ICL was at one stage under considerable pressure to join this triumvirate, but resisted, and consequently remains alive and well—and profitable. □

WHEN the Loch Ness Monster ostensibly raised its ugly head (see picture) with the support of Sir Peter Scott, widespread public interest was inevitable. What was far less certain was the reaction of the scientific community. As it turns out, Scott and his colleague Robert Rines must be reasonably content.

Their original plan was to present at a two-day scientific symposium the full evidence which they felt supported the existence of large animals in Loch Ness. The outcome, they hoped, would at least allow the existence of the animal to be given the benefit of the doubt. They also wanted it officially named in order to have it legally protected as an endangered species. An attack of cold feet gripped the organisers of the symposium, however, and they withdrew their support.

The only forum left for the presentation of evidence was a meeting in the Grand Committee room at the House of Commons—a room, some would say, that is steeped in the history of monstrous debate. It was held on the evening of December 11 before representatives of the public, the press, and the scientific community, along with Members of Parliament understandably seeking relief from the second reading of the Armed Forces Bill, which was making Mr Wellbeloved less endearing than Nessie.

The bulk of the meeting was devoted to presentations by members and associates of the Academy of Applied Science—a 'non-profit, scientific and educational corporation' lodged at the Boston home of its president, Robert Rines. The academy has been investigating Loch Ness every summer for the past five years.

The optical and sonar systems used and the site chosen for the attempt to obtain underwater photographs of the monster were both presented in dazzling detail. Much was then made of the 1972 photographs which, after computer enhancement, showed 'flipper-like objects'. But when it came to the expected climax of the 1975 photographs, Rines' presentation was so modest that he nearly blushed. No

attempt was made to identify the objects shown. That, said Dr Rines, was for qualified biologists to do—not himself.

Three biologists handily placed on the platform then stated their beliefs that the photographs contained various images suggestive of flippers, a body

Nessiteras skeptyx



and a head, all of which indicated the presence of large animals in Loch Ness. In that they were further supported by a statement from Professor A. W. Crompton of Harvard. None of the biologists, however, was prepared to try to identify the animal.

The discussion period that closed the meeting provided few fireworks. Most of the questions were on a scientist-to-scientist basis; they ranged from the problem of whether the animal would be air-breathing to queries about size controls for the objects photographed. Critical comments attained various levels. Hugh Fraser MP said that judging by his disappointing fishing experience in Loch Ness, the salmon population of 13 million claimed earlier in the evening was a gross exaggeration. He did not proffer an alternative explanation of his performance, relating to the likely appetites of any animals in the Loch. David Attenborough suggested that the 1972 flipper-like objects might actually be the fin of a fish. And that, it seemed,

was the cue for the heavy artillery from the British Museum to open fire.

Dr Gordon Sheals supported the view that the flippers were fish fins, and that their estimated size was "wildly" miscalculated. When that was disputed and further claims were made for the presence of a large animal, he decided it was time to broaden the attack. There was, he said, no evidence to support the view that the same object was shown in each photograph, nor to suggest that any of them showed a living object. Furthermore—and this was the body blow—it was most regrettable that *Nature* had published the article by Scott and Rines.

Another sceptic from the British Museum, Dr Humphrey Greenwood, conceded what several more sympathetic voices had already suggested—that there was a sufficient case for the evidence now to be published in full detail so that it could be properly scrutinised. The delighted Dr Rines clearly felt the battle was half won. He was at least being taken seriously.

Less serious was the response of the mass media. By and large the photographs were presented with a healthy dose of scepticism to accompany them and some astonishing artist's impressions of what they showed. The more enterprising journalists managed to winkle out people with something they thought approached a knowledgeable view. Unsurprisingly, the claims were as diverse as they were knowledgeable. One view was that the photographs actually showed parts of a Viking ship. Another, on television, was that the "head" was of a scuba diver wearing his breathing apparatus back to front. The grateful monster-following public also learned that the British Bacon Curers' Federation was to organise a hunt for Nessie by hot air balloon.

If the eventual truth of the monster-hunters' claims is still breathlessly awaited, at least they, though perhaps not their prey, have finally come in from the cold. That may be the epitaph on this episode. In the next one, the natural history of Loch Ness may even become a respectable line of study.

international news

Exit the Dragon?

by Roger Woodham

THE slight chance that the Dragon international high temperature reactor might be saved at the eleventh hour by the EEC Council of Ministers slipped away last week when the matter failed to come up for discussion at all. The decision taken the previous week in Brussels therefore stands, and the Dragon will be killed off—though not immediately dismembered—on March 31.

This brings to an end a three-month period during which optimism and pessimism over the future of Dragon fluctuated weekly, with the US Energy Research and Development Administration (ERDA) expressing interest in the project without putting any money on the table—or rather on the telex. Any UK agreement to continue the project eventually came to depend on some form of financial involvement by ERDA, and when this failed to materialise, the EEC, representing several of the participants in the project and therefore having the whip hand, decided to finish the project off.

The only real concession achieved in the past few weeks was a stay of execution of three months, from December to March. Fortunately the majority of the 300 or so people who work on Dragon are seconded from the UK Atomic Energy Authority (UKAEA) or its equivalent in the other member countries, or from industry, so they will simply return to their own organisations.

What happens between now and March 31? Certainly nobody is going

to start taking the reactor to pieces—the UKAEA doesn't really know how to dismantle a reactor safely yet—and it will simply be mothballed. Late last week the European Parliament in Strasbourg criticised the decision and called for the reactor to be saved, and there is always the chance, of course, that ERDA, which by all accounts has been showing more definite interest even during the past week or so, may come up with a concrete proposal. But the possibilities all seem rather remote now.

Nothing definite emerged from a debate in the British House of Commons last week either. Replying to critical questioning, Alexander Eadie, Under-Secretary for Coal at the Department of Energy, said that the government was conscious of its special place as host, and did not want the project ended if other participants wanted it to continue. But in that case, why not leave more time for alternative plans to be worked out properly?

It looks as if the Dragon on Winfrith Heath will stand as a monument to 16 years of work which eventually got out of step with the UK's nuclear aspirations. □

University money plea

IN the politest way possible, the Committee of Vice-Chancellors and Principals of UK universities has asked Mr Fred Mulley, the Secretary of State for Education and Science, to put university finances back on to a firmer footing.

The recently issued statement "Universities in a period of economic crisis"

asks the government to do three things:

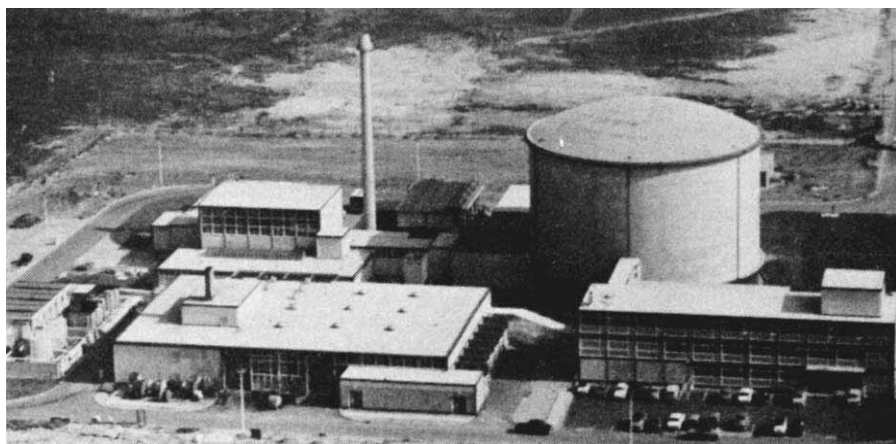
- provide a planning perspective and a 'real terms funding commitment' extending beyond a year;
- assure universities that beyond the short-term crisis they will be able to resume development programmes with support "at a level in keeping with their national and international standing and commensurate with the burdens they have shouldered", and
- give a commitment that long-term planning and financing arrangements will be restored.

The committee reminds Mr Mulley that universities have been working on reduced resources in real terms for the last eighteen months and yet even so managed to increase student numbers by 7,000 in 1974 and 10,000 in 1975. The number of full-time students in British universities is now 263,000. More growth is forecast in the near future as the population of 18-year-olds has started to increase again and there is a "welcome surge" of applications from women.

Grants to universities are channelled through the University Grants Committee, and academic salaries comprise a little over 50% of university expenditure. Increases in salaries are guaranteed by the government, but the other half of recurrent expenditure (salaries of other staff, maintenance, supplies and so on) is compensated against inflation in rather a strange way. A costs index is used and the government may choose to make appropriate compensation in arrears.

In the calendar-year 1973 costs rose 12%, and the government chose not to supplement at all for the academic year 1974–75. Meanwhile cost inflation was running at 29% in 1974. As a result of devaluations, the government later supplemented to the tune of £19 million for the year 1974–75 but, universities claim, this represented only a third of the sum necessary to maintain the grant in real terms.

In response to this universities had to make their own economies, by freezing staff posts (500 are now frozen), by letting non-academic staff go, and by saving of libraries, fuel, maintenance and so on. In terms of physical facilities, the universities now believe they have done all they can reasonably hope to, and in terms of staff cuts, they are now up to a student:staff ratio of 10:1. "Lasting damage would shortly ensue", they claim. □



The Dragon reactor: now a monument?

Cottrell call for coal

A FORMER chief scientific adviser to the British Cabinet has involved himself in the continuing debate on the UK's energy prospects by publicly criticising the government for failing to appreciate the central importance of Britain's coal resources. In a letter to a leading newspaper last week Sir Alan Cottrell expressed concern that the country will face a serious energy deficiency when North Sea oil reserves are largely depleted in around 15 years time.

He called for an increased programme of government research into the extraction and efficient utilisation of coal so that Britain can fill the post-1990 energy gap, arguing that the country cannot hope to meet its future

energy requirements with nuclear power alone. It is estimated that by the year 2000, when nuclear output will only have doubled, Britain will face an energy gap equivalent to 20 times its present installed nuclear capacity.

Sir Alan says the present level of capital investment is insufficient to finance nuclear development on the necessary scale, and the recent cut-back in the nuclear industry has resulted in a decline in the available constructional capacity. He adds that there is also a lack of public support for an expanding nuclear programme, and that a problem of uranium fuel shortage will arise.

He sees a fuller exploitation of British coal reserves as unavoidable, and argues that economic means of extracting coal from difficult deposits,

almost certainly by remotely operated machines, must be developed; and that techniques for producing high-grade, low sulphur fuel oil and gas from coal must be advanced. Research into both of those problems is already well under way but Sir Alan says the programme will have to be stepped up if Britain is to come within range of its target in the time available.

The letter nevertheless endorses the recent enjoiner from the UK Royal Commission on Environmental Pollution that the government press ahead with the construction of a demonstration fast breeder reactor. And EEC policy regarding fusion reactors comes in for a timely sideswipe: according to Sir Alan, fusion reactors will not be ready for many years yet on the "presently dilatory time scale". □

REGIONAL economic communities brought into being for the mutual benefit of their members will always experience difficulty deciding on the location of cooperative projects. The EEC is no exception, and last week provided a nice example of the problems involved.

The project was the Community's ambitious thermonuclear fusion programme for 1976-80, and the issue was the location of its acknowledged centrepiece, the large experimental rig called the Joint European Torus (JET). The result, at a Council of Ministers meeting in Brussels devoted to collaboration in scientific research, was that Italy blocked the programme—and the reason was that it wanted the project sited at the Nine's Joint Research Centre at Ispra in Northern Italy.

The European Commission, whose plans for this fourth thermonuclear fusion programme involve an expenditure over the four years of some £250 million, will not make a formal recommendation on the location until the new year, but has indicated a preference for Ispra.

Germany and France have had reservations, however, while Britain has pressed for the project to be sited at the Culham Laboratory of the UK Atomic Energy Authority. Unlike the other eight countries, Italy was not prepared to endorse the expenditure without a commitment to a location.

The Italian veto—for that is what it amounts to—marks a setback to a project which offers the possibility in the long term of an independent source of energy within the Community. Thermonuclear fusion is arguably a key to Community energy supplies beyond the end of the century, and marks one branch of re-

search in which Europe is abreast of both the USA and the USSR. Herr Guido Brunner, the Brussels Commissioner responsible for scientific research, describing the delay as a "great error", warned that the Community's strong position in fusion research was in danger of being lost without an early go-ahead on the programme.

EEC diary

● Further signs of movement towards a European energy policy surfaced ahead of last week's Paris meeting between oil producers and consumers, for which the EEC finally acquired a mandate after the procedural difficulties that dogged the Rome summit meeting.

Apart from agreeing that average community oil consumption in the next two years should be a little below the 1973 level, a meeting of the Nine's foreign ministers received a progress report from the Commission on a directive under which member countries are obliged to keep an emergency oil reserve equivalent to 90 days' supply.

The requirement was fulfilled in June this year—when stocks were sufficient for 95 days' consumption. Although down from 101 days in January, the main problem was that the level was only for the Community as a whole. Some countries—West Germany, Italy, Holland, Belgium and Ireland—had failed to introduce the necessary legal measures to give effect to the directive, which came into force at the beginning of the year.

The Commission now intends to

take legal action against these countries, although it admitted there were difficulties in stock building because of the large amounts of capital it immobilised and the balance of payments burden it imposed on the countries concerned.

● In the UK a recently published House of Lords Select Committee report criticises a Commission resolution calling for the standardisation of nuclear safety regulations within the EEC. The report, which comes only months after the committee strongly criticised Commission proposals for a Community energy policy, also attacks accompanying proposals that approval procedures for nuclear plant should be centralised in Brussels and that a body should be established to coordinate discussion of nuclear safety.

If the proposals are adopted, existing safety standards could suffer, the report says. The committee's chairman, Lord Lauderdale, said this was because the Brussels administration, with no real experts of its own, would preoccupy experts already working on safety problems.

The report says that to standardise safety proposals is impractical because "different types of reactor are in use, and design is developing". Standardisation will be "social rather than strictly commercial", and centralisation of approval procedures is seen as dangerous because "the ability to make direct representations to accessible elected representatives at a public enquiry is a valuable safety valve which would be lost". The establishment of a body to act as a catalyst for the international discussion of safety measures is, according to the report, unnecessary in view of the plethora of organisations already in existence.

LEGISLATION to re-establish a science policy office in the White House, which has long been a goal of the scientific leadership in the United States, has encountered an unexpected roadblock in the Senate. Having swept through the House of Representatives a few weeks ago with scarcely a murmur of dissent, the bill is now stalled in three separate Senate committees chiefly because of growing disagreement about whether or not Congress should specify in detail the office's functions and areas of responsibility.

The essence of the matter is that Senator Edward M. Kennedy, who is the chairman of one of the subcommittees dealing with the bill, believes that the science policy office should play a strong role in military matters as well as domestic science policy affairs, and he wants to write strict provisions into the bill to ensure that is the case. The House-passed version, in contrast, would leave the President virtually sole responsibility for deciding how the office should function and what its areas of responsibility should be. Unless its role is spelled out in some detail, Kennedy is concerned that the office would be an ineffectual junior partner in the White House structure.

Kennedy's views are said to be shared by Senator John Tunney, who chairs one of the other subcommittees concerned with the legislation and, to a lesser extent, by Senator Frank Moss who chairs the third committee. That is a pretty solid front, and since the matter is very low in the consciousness of most other legislators, there will be little pressure for them to change their minds at least from within Congress.

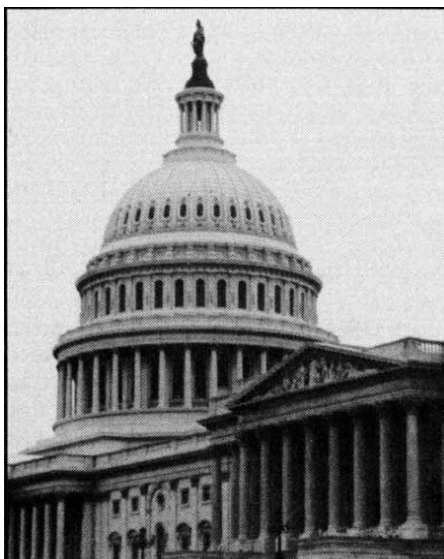
The White House, however, is opposed to the notion that Congress should write a detailed prescription for an office which will be functioning at the President's elbow, and it strongly favours the House-passed version of the bill. The House members concerned with the matter are also of the opinion that the President should have considerable latitude in deciding how the White House staff should function, and have indicated that they are prepared to stick by their bill. They have argued, for example, that the influence of the science policy office will depend in large measure on whether or not it can function effectively in relation to other White House units and it should therefore be left to establish its own working relationships.

Although it is possible that the senators could compromise, and hammer out a measure acceptable to the White House and the House of Representatives, the prospects at present are uncertain. In any case, the start-up date for the office has receded considerably, and it now seems likely that

it will be in place just as the Presidential election is in full swing. With President Ford's chances of re-election uncertain, it would be difficult to recruit good staff to the office, and nothing dramatic could be expected to emerge from it at least until the election is over.

Washington seen

by Colin Norman



● Another contentious issue is about to emerge in the bitter nuclear power struggle in the United States. On January 19, the Nuclear Regulatory Commission (NRC) will send Congress a report on the feasibility of grouping together nuclear power stations, and perhaps other nuclear facilities, on a few sites instead of scattering them around the country. According to a draft of the report, written by the NRC staff, such nuclear energy centres are deemed both economically advantageous and practical.

The matter is likely to be controversial, however, because such centres would raise some severe environmental problems, although they could help to mitigate some other concerns about nuclear power generation.

The study, which was ordered by Congress when it wrote the bill establishing the Nuclear Regulatory Commission, concludes that nuclear centres, with up to 20 power stations each, could cut the costs of building power plants by about 15%. Since individual plants are expected soon to cost about \$1,000 million, such savings would be substantial.

But the environmental problems associated with nuclear energy centres would also be substantial. A 20-unit site, for example, would encompass about 40 square miles, compete for scarce water supplies with other users, such as cities and agriculture, and disperse so much waste heat into the

environment that local weather patterns could be affected.

One frequently claimed advantage for grouping power stations, fuel fabrication and reprocessing plants together on the same site is that, when the nuclear industry begins using plutonium as a reactor fuel, having various facilities side-by-side will greatly reduce the risks that atomic bomb ingredients could be stolen in transit. But the study pours some cold water on that idea, suggesting instead that conventional safeguard procedures will be sufficiently effective to take care of the problem.

That particular statement is interesting in light of the fact that NRC has delayed making a decision on whether or not the nuclear industry should be allowed to recycle plutonium as a reactor fuel, precisely because it is not yet convinced that safeguard measures are adequate.

Be that as it may, the study is likely to generate a good deal of debate within Congress this year. It will also rekindle debate at the state level, since several state governments are considering adopting legislation to group power plants together.

● Spurred on by a highly critical report of the Food and Drug Administration's regulation of a ubiquitous food additive, Red Number 2, Senator Gaylord Nelson has proposed legislation which could force several food colourings off the market because of doubts about their safety.

Nelson, a liberal Senator from Wisconsin, who has long been a thorn in FDA's flesh, wants to repeal a provision of the food and drug laws which at present allows scores of food additives to be marketed while FDA collects data on their safety. The provision, which was written into the law in 1960, essentially allowed FDA 30 months to collect test data on additives that were then on the market, and determine whether or not they should be declared safe or banned. Fifteen years later, FDA has still not made up its mind about 90 colouring additives which were being marketed in 1960.

Among those additives is Red Number 2, which in 1973 was consumed at the staggering level of 1.1 million pounds in the United States. For years, there have been conflicting reports of health hazards associated with Red Number 2, including carcinogenicity, teratogenicity, and gonadal atrophy, and a few weeks ago, the Congressional General Accounting Office issued a sharply worded criticism of FDA's delay in deciding whether or not the additive is safe while children consume it in copious quantities in soda pop and candies. Nelson's bill would force FDA to make up its mind about those additives.

news and views

MANY countries bordering the Pacific contain rocks older than any preserved in the floor of that ocean where little material exceeds 200 million years in age and most is much younger. Yet the Pacific Ocean appears to have formed much further back in time and may have existed in the Precambrian, as long as 1,000 million years ago. Valuable clues to the early history of the Pacific can occasionally be picked up through study of old rocks preserved on land around its margin. Recent work by two members of the British Antarctic Survey on islands of the Scotia Arc has brought particularly interesting evidence to light. In this issue of *Nature* Smellie and Clarkson (page 701) describe new clues to possible conditions in Palaeozoic times along part of the south-east edge of the Pacific.

From studies around Scotia Arc, the 4,000 kilometre long ridge (in part submerged, in part exposed as groups of islands) which joins South America and Antarctica, they conclude that in Upper Palaeozoic times Pacific Ocean floor was being subducted and destroyed in that region.

They base their conclusions on their recent discoveries of the mineral glaucophane, a mineral found in metamorphic rocks which have been subjected to unusually high pressures and one which characterises many of the well-established subduction zones formed more recently in the history of the Pacific. Smellie and Clarkson found Palaeozoic glaucophane schists in the South Shetlands not far from the Antarctic Peninsula. Coupled with earlier discoveries of similar rocks

Antarctic jigsaws

from J. Sutton

among material dredged from the vicinity of the northern arm of the Scotia Arc, the new finds suggest the presence of a Palaeozoic glaucophane schist belt along which Pacific floor may have been subducted. Offset from part of this belt and roughly parallel to it a belt of rather different metamorphic rocks and intrusions has been identified on the Antarctic Peninsula through the work of many members of the British Antarctic Survey. The nature of these rocks suggests a Palaeozoic belt formed by rather higher heat flow than that indicated by the glaucophane schist belt. Smellie and Clarkson suggest that the two belts could be compared with the paired metamorphic belts which are a familiar feature of many younger terranes around the Pacific.

As a rule each pair comprises a metamorphic belt whose mineral make-up indicates a rather low heat flow parallel to a second belt further from the Pacific, formed by higher flux of heat. The newly identified Antarctic belts follow this pattern and suggest there was an active Palaeozoic plate boundary between the south-east Pacific and the land masses of what is now Western Antarctica and southernmost South America.

That belt of subduction may well have preceded the opening of the South Atlantic and may have been active at a time when South America and Africa still formed a single block. Whether that block had separated from Antarctica at the period is uncertain. The Palaeozoic rocks may have formed along the Pacific coast of Gondwanaland or have been part of an early island arc—a proto-Scotia Arc linking Antarctica with the South American–African continent.

Solving such problems entails careful examination of the older rocks preserved at intervals along the Scotia Arc. Recently, I. W. D. Dalziel has reviewed progress made between 1969 and 1975 on the Scotia Arc tectonics project mounted by Lamont (*Antarctic Journal of the United States*, 1975). There Dalziel summarises recent developments in the model for the evolution of the Scotia Arc earlier proposed by himself and Elliott, (*Nature*, **233**, 246–252; 1971). Dalziel and his colleague suggest that the Scotia Arc region was deformed in a Gondwanian orogeny in the early Mesozoic. From studies of Mesozoic sediment within the arc, notably on South Georgia and from within the South American continent they are able to suggest the Gondwanian orogeny resulted either from a collision of an island arc with the main part of Gondwanaland or from subduction beneath the margin of that super continent. Bit by bit the early history of the continental crust now widely dispensed along the Scotia Arc is emerging and the jigsaw of the present day fragments can be more confidently put back into place. □

THE role of microtubules in the strategy of the cell is a very important one. The question of what functions they are actually involved in, is more difficult to answer and very much a subject of speculation. The main evidence for the participation of microtubules in such cellular activities as transport and secretion of cell constituents rests on inhibitor studies (for a recent review see Roberts *et al.*, *Prog. Biophys. molec. Biol.*, **28**, 371; 1974). These drugs can disaggregate microtubules by binding to tubulin, both in the cell and *in vitro*, and this is assumed to be their mode of action in inhibiting cellular processes. To prove this conclusively, however, is

Membrane-bound tubulin: fact or artefact?

from Mike Jacobs

no easy matter. Colchicine, for example, inhibits the secretion of plasma proteins from the liver at the levels predicted by its binding constant to tubulin. Inhibition is accompanied by a dramatic accumulation of protein in the Golgi-derived secretory vesicles (Redman *et al.*, *J. Cell Biol.*,

66, 42; 1975; Le Marchand *et al.*, *J. biol. Chem.*, **248**, 6862; 1973). Other obvious metabolic parameters are unaltered and colchicine analogues and drugs that do not bind to tubulin are ineffective.

The morphological evidence is not so good. Reports differ as to whether microtubules are depolymerised when secretion is inhibited. There are few microtubules in liver cells, and their orientation suggests no obvious role in secretion. Other studies of secretory cells often tell the same story; there is sparse anatomical evidence to support a mechanism by which microtubules transport 'packaged' molecules.

The finding of tubulin and colchicine

binding in the membranous fraction of cells, may provide an alternative site for colchicine inhibition. Substantial amounts of tubulin are found in the post-synaptic functional lattice (Walters *et al.*, *Nature*, **257**, 496; 1975) and in synaptic membranes and vesicles (Blitz *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4144; 1974). There is little doubt that tubulin is present in these membrane preparations, as the protein was carefully characterised by SDS gel electrophoresis and peptide mapping. The main difficulty is to be sure that the soluble cytoplasmic tubulin is not trapped or adsorbed to the membrane during preparation.

Using thyroid and neuronal membranes, Bhattacharyya *et al.* (*J. biol. Chem.*, **250**, 7639; 1975) attempted to resolve this problem. By adding radioactive tubulin the contamination of these membranes by cytoplasmic tubulin was estimated as 0.1%. Nevertheless, without knowing the relative specific activities of the membrane and soluble tubulin, it is still possible that this small contamination represents a significant proportion of the membrane protein. Another problem is to consider what radioactive tubulin to add, as a label for soluble tubulin. 6S tubulin may not equilibrate with the ring form (36S) of tubulin or fragments of microtubules (Weingarten *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1858; 1975).

In these circumstances a better control may be to add radioactive microtubules. Another method of assaying the putative tubulin is by colchicine binding. In thyroid and neuronal membranes prepared by two different methods, colchicine binding activity is present far in excess of contaminating soluble tubulin. So the latter may not be such a problem. Colchicine binding to membranes by itself is not surprising since the molecule is so hydrophobic. What suggests that this binding site is indeed tubulin, is its respective affinities for colchicine and its analogues, and vinblastine, which are identical to those of soluble tubulin. Furthermore, the colchicine binding site can be solubilised with an enrichment in both its activity and in a protein co-migrating with cytoplasmic tubulin on SDS gel electrophoresis. The membrane colchicine site is more heat stable and has a higher optimum binding temperature than soluble tubulin, but reverts on solubilisation. Soluble tubulin in high sucrose shows a similar effect which is explained as due to preferential hydration preventing the protein unfolding (Lee *et al.*, *Ann. N. Y. Acad. Sci.*, **253**, 284; 1975). Such an explanation for the membrane-associated tubulin would imply a specific interaction between the two components. Further doubt as to the

QSO redshifts and the steady state cosmology

JOHN GRIBBIN (*Nature*, **257**, 540; 1975) has ably summarised the work by one of us (P.K.D.) which appeared recently (*Mon. Not. R. astr. Soc.*, **172**, 623; 1975) on the subject of the relationship between angular diameters and redshifts in Hoyle-Fowler type models of QSOs. Gribbin's ability as a mind-reader, however, must be seriously questioned when he writes in his preamble: "... although Das does not mention the steady state theory, it seems a reasonable assumption that any colleague of Narlikar's who discusses possible non-cosmological contributions to the redshifts of QSOs has the concept not too far towards the back of his mind."

While discounting that any such 'unholy' thought was at the back of our minds in our discussions of the Hoyle-Fowler objects (*Mon. Not. R. astr. Soc.*, **171**, 87; 1975), we would like to take this opportunity to point out that too much importance is being given by several astronomers and science writers (*New Scientist*, July 3, 4; 1975) to any connection between the steady state theory and the non-cosmological theory of QSO redshifts.

It is true that observations indicate that strong evolutionary effects are at work if the QSO redshifts are wholly cosmological in origin. It is also true that such evolutionary effects would be against the simple steady state

hypothesis. Thus a theory of non-cosmological origin of QSO redshifts is probably necessary for the survival of the steady state theory. But here the connection between the two ends.

For example, even if the QSOs turn out to have an appreciable non-cosmological component in their redshifts, this does not validate the steady state theory which still faces other observational hurdles like the microwave background. Nor does the disproof of a particular non-cosmological redshift hypothesis in a single QSO—for example, the gravitational redshift (Wampler, *et al.*, *Astrophys. J.*, **198**, L49; 1975) in the case of 3C48—particularly embarrass the steady state theory.

Indeed, regardless of any cosmological prejudices it is pertinent to ask: "Is the redshift of a QSO due to the expansion of the Universe alone?" A fair assessment of the present data does not permit an unequivocal affirmative answer to this question. Even the overall problem of the structure of a QSO still remains unsolved. Under these circumstances even theoreticians sympathetic to the steady state cosmology may work on models of non-cosmological redshifts for reasons not primarily concerned with defending that cosmology.

J. V. NARLIKAR
P. A. DAS

identity of the colchicine binding site on membrane should disappear when it can be definitely attributed to the solubilised membrane tubulin.

A role suggested for membrane-associated tubulin is as an anchor for cell surface receptors (Berlin *et al.*, *Nature*, **247**, 45; 1975; Edelman *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1442; 1973). Whether it does this in its unpolymersised form or as microtubules must be investigated. Then there are the other three fibrous protein, actin, myosin and spectrin, claiming association with membranes. How all these proteins function remains an exciting and intriguing question for the future.

Lasers and the Lamb shift

from Peter Knight

THE investigation of atomic structure by conventional spectroscopic techniques (for example, excitation using electron-impact in a discharge tube and resolution

of the emitted radiation with interferometers) is severely limited by Doppler broadening and consequently relies heavily on a precise understanding of the lineshape of the emitted radiation. To see the subtle effects of current interest within Doppler broadened spectral lines, with widths typically of the order of GHz, the spectroscopist is forced to the limits of available technology. This is especially true of that most "fundamental" of atoms, the hydrogen atom, which, being the lightest atom suffers most from Doppler broadening. The arrival of the tunable laser in atomic physics has, however, totally revolutionised optical spectroscopy. This long-awaited revolution is perhaps best characterised by a series of beautiful experiments on atomic hydrogen using dye laser excitation performed by T. H. Hänsch and coworkers at Stanford. In a recent paper (*Phys. Rev. Lett.*, **35**, 1262-1266; 1975) Hänsch, Lee and Wallenstein report measurements of the isotope shift of the 1s-2s transition in atomic hydrogen and deuterium, and the Lamb shift of the 1s ground state of

hydrogen, both very small effects compared with the (optical or ultraviolet) transition frequency. As a demonstration of the power of their new technique, they report improvements of several orders of magnitude over previous measurements of this isotope shift.

The key feature is the ability of the laser spectroscopist to overcome the Doppler broadening of spectral lines. The monochromaticity of the laser allows selective excitation of atomic excited states. At the same time the laser intensity is high enough for non-linear absorption effects to be exploited: one of these is saturated absorption. A laser beam, with a linewidth much less than the Doppler width of the spectral line of interest, interacts not with all atoms in the absorption cell, but with a set of atoms with resonance frequencies (and therefore velocities) in a narrow range around the laser frequency. An intense beam of laser radiation will excite this set of atoms: it saturates the gas medium and burns a "hole" in the velocity distribution of ground-state atoms. In saturated absorption spectroscopy the output of a laser is split into a weak probe beam and a stronger, chopped saturating beam of the same frequency which are sent in nearly opposite directions through the absorbing gas. If the laser frequency is close to an atomic resonance frequency, the saturating beam bleaches a path for the probe and decreases its absorption, but only for that group of atoms with effectively zero velocity along the laser beam-path. Moving atoms see the two beams as of different frequencies, and no such bleaching occurs. In this way the Doppler effect is almost eliminated. The saturation spectrum is obtained by varying the laser frequency and recording the change in the weak probe beam due to the inhomogeneous saturation created by the strong bleach beam. The theoretical limit to the linewidth is then governed by the natural lifetime of the excited state.

A second technique to eliminate Doppler broadening is to use two-photon absorption. This time the laser is split into two counter-propagating beams of equal intensity. This may be achieved merely with a mirror at one end of the cell to reflect the incident light back down the cell. If the laser frequency ω is approximately one-half of an atomic resonance frequency ω_0 , an atom can be excited by the absorption of two photons, one from each oppositely travelling wave. An atom of laboratory velocity v sees, in its rest frame, two beams of frequency $\omega(1 - v_x/c)$ and $\omega(1 + v_x/c)$. At resonance, $\omega_0 = \omega(1 - v_x/c) + \omega(1 + v_x/c) = 2\omega$, independent of the velocity of the atom. Therefore Doppler broadening is eliminated if the atom absorbs two photons propagating in opposite directions, and again the natural lifetime of the excited state is the limiting factor in the linewidth. The two-photon absorption



A hundred years ago

THE ravages of the Phylloxera among the vines have caused many attempts to be made to discover a new kind of beverage which might take the place of the grape. The Marquis de Ville-neuve reports that in China a *pseudo* wine called *Tsien-ia* is much used, which is concocted from a preparation of four plants, common in that country, and mixed together in certain proportions. The plants are dried and powdered, and made into a paste, which is sold in the form of balls or squares at the rate of about 3d. a pound. One square or ball will make several pints of a fermented liquor, pleasant to the taste and much resembling wine, which is much sought after by Europeans and others living in China.

from *Nature*, 13, December 30, 1875;

signal has contributions from all atoms in the cell, irrespective of their velocities. This technique also makes possible Doppler-free spectroscopy of $s \rightarrow s$ or $s \rightarrow d$ transitions, not possible by one-photon saturated absorption.

Hänsch and his co-workers have simultaneously used two-photon absorption and saturated absorption Doppler-free spectroscopy in their hydrogen work.

A pulsed dye laser output at $\sim 4,860 \text{ \AA}$ in 10 ns pulses of linewidth 120 MHz, is split into two beams. Noting that the crude Bohr theory predicts that the $n = 1-2$ interval is four times larger than the $n = 2-4$ interval, they contrive to frequency-double one beam (so that its wavelength is now $\sim 2,430 \text{ \AA}$) and excite the $1S_{1/2}-2S_{1/2}$ Lyman- α transition at $1,215 \text{ \AA}$ in a cell of hydrogen or deuterium atoms, by two-photon absorption. Since an isolated atom in the $2S_{1/2}$ state has a lifetime of $\sim 0.14 \text{ s}$ this transition could potentially be measured to very great accuracy. At the same time the second beam is used in a saturated absorption spectrometer to measure the $2P_{3/2}-4D_{5/2}$ transition interval at $4,860 \text{ \AA}$ in a separate cell. By varying the laser frequency, therefore, both transitions can be simultaneously recorded. The measurements are not made in the vacuum ultraviolet, liquid air cooling of the discharge and the use of large gratings is avoided, yet great accuracy is possible.

If the Bohr theory were correct, the two observed transitions would occur at exactly the same dye-laser frequency. The

fact that they are displaced is due to quantum electrodynamic effects (principally the Lamb shift of the various levels), relativistic effects, and partly to nuclear structure effects. The measured displacement between the two transitions gives directly information about the size of these effects without the need for absolute wavelength calibrations or an ultra-precise value of the Rydberg constant. By comparing the experimental and theoretical line separations, Hänsch *et al.* deduce a $1s$ Lamb shift of $8.20 \pm 0.10 \text{ GHz}$ for H, and $8.25 \pm 0.11 \text{ GHz}$ for D, which agree within their error limits with the theoretical values. At the same time they obtain a $1s-2s$ isotope shift in atomic hydrogen and deuterium of $670.933 \pm 0.056 \text{ GHz}$, in fair agreement with the theoretical value, but representing an improvement of several orders of magnitude over previous (traditional pre-laser) measurements. To appreciate the leap in accuracy made possible by Doppler-free laser techniques, this measured isotope shift should be compared with the hydrogen Lyman- α line Doppler width of $\sim 6 \text{ GHz}$.

To strike fear into the hearts of theoreticians, they point out that much more accurate experiments are possible using two-photon absorption in the $1s-2s$ transition, and two-photon absorption from a GaAs diode laser, whose second harmonic is locked to the dye-laser frequency, driving a $2s-4s$ transition. Given the lifetimes of the various participating levels, they feel the ultimate accuracy of such proposed optical experiments could exceed recent high-precision radiofrequency measurements of the $2s$ Lamb shift, and even that of present day theory of the Lamb shifts.

These elegant experiments of Hänsch and coworkers emphasise the part that the tunable laser has played and will play in precision spectroscopy, a role that it has only recently assumed yet which has already produced a qualitative change in the direction of atomic physics. $\square$

Heat flow on different scales

from Peter J. Smith

UP to the late 1960s, heat flow studies were generally regarded as interesting and potentially important but of little immediate use. Their underlying importance was acknowledged because heat plays a part in almost every conceivable terrestrial process, but the promise they held out to illuminate the nature of such processes was difficult to realise. Part of the problem was that data coverage was poor and there were large areas of the world with few or no

heat flow observations. Such areas still exist, unfortunately, but they present fewer difficulties than before as emphasis has now shifted somewhat from global analysis towards the application of heat flow data to component parts of the global tectonic system, whether they be oceanic ridges, subduction zones or even whole oceans.

A good example of this trend towards smaller-scale phenomena is provided by R. N. Anderson (*J. geophys. Res.*, **80**, 4043; 1975) who has used heat flow observations to elucidate the nature of the Mariana trough. The interpretation of the marginal basins which lie behind island arcs in the western Pacific has long been a matter of debate. The suggestion that such basins are extensional features was made as long ago as 1954 and has been repeated many times since, although it was left to Karig (*J. geophys. Res.*, **75**, 239; 1970 and *Geol. Soc. Amer. Bull.*, **82**, 323; 1971) to provide the detailed geological evidence to support it. Karig showed that marginal basins are bounded by steep normal fault zones, that the bounding arcs are covered with thick sediment whereas the basin interiors are almost sediment free, and that in some cases within-basin fault block morphology can be traced onshore as a graben system.

These and other results led Karig to conclude that marginal basins in general and the Mariana trough in particular were formed by recent intrusion and crustal extension—a view now widely, though perhaps not universally, held. But even if this is accepted, it still leaves open the question of just how the extension occurs. Karig himself proposed extension by dyke intrusion along the central axial high of the Mariana trough, although he also produced evidence suggesting that the rate of extension decreases towards the edges of the basin. In a sense, this is an intermediate view between that of Sclater (*J. geophys. Res.*, **77**, 5705; 1972), who proposed a mechanism akin to seafloor spreading but with the production of a thinner than normal crust, and that of Hasebe *et al.* (*Tectonophysics*, **10**, 335; 1970), who suggested a slow diapiric rise of magma ('incipient intrusion') throughout the basin.

There are no regular magnetic anomalies over the Mariana trough of the type usually taken to support seafloor spreading; but this is not conclusive evidence against spreading because any anomalies would probably be disrupted by the rough topography and many transverse fracture zones. It is at this point therefore that heat flow should be able to settle the matter, for the different models proposed give rise to different heat flow regimes. Unfortunately, most heat flow values obtained from

the Mariana trough so far have been abnormally low, thereby appearing to support none of the models and creating what Watanabe *et al.* (*Tectonophysics*, **10**, 205; 1970) have called an "anomalous, difficult situation".

But 21 heat flow measurements now reported by Anderson tell a different story. With one exception, both new and old values on the flanks of the axial high are indeed low—lower than the 1.1 h.f.u. mean for the oldest parts of ocean basins and in one case zero—irrespective of whether the stations were in sedimentary ponds, on flat sedimentary blankets or on local topographic features. On the axial high itself, however, high heat flow was observed—in one case 8.45 h.f.u. In short, with sufficient data coverage the distribution of heat flow across the Mariana trough broadly resembles that across an oceanic ridge. Anderson's conclusion, therefore, is that the trough is being produced by seafloor spreading with a narrow zone of magnetic intrusion centred on the axial high.

The wide range of environments in which heat flow over the flanks of the axial high was universally low led Anderson to attribute the low values to hydrothermal circulation. Fluid circulation also plays an important part in Albarede's study (*Earth planet. Sci. Lett.*, **27**, 73; 1975) of the linear relationship between heat flow (q) and surface heat production (A) in continental plutonic rocks, discovered by Birch *et al.* (in *Studies of Appalachian Geology*, Interscience, 1968). The simplest interpretation of this relationship ($q=a+bA$, where a and b are constants over large areas known as 'heat flow provinces') is that radioactivity is constant to a depth b and zero below. As Lachenbruch (*J. geophys. Res.*, **73**, 6977; 1968) pointed out, however, this interpretation is inconsistent with differential erosion within a heat flow province; instead, radioactivity must decrease exponentially with depth.

But how does this exponential dependence arise physically? Various suggestions have been put forward ranging from the effects of fractional crystallisation of cooling magmas to equilibrium established by gravitational forces. There are objections to all of them; and so Albarede has developed a new model, based on the known mobility of the radioactive elements U and Th, in which a redistribution of radioactive elements is achieved by migration of water and other fluids through the magma during crystallisation and cooling.

Meanwhile, the global picture is not being ignored completely, for Chapman and Pollack (*Earth planet. Sci. Lett.*, **28**, 23; 1975) have carried out an important new analysis of all heat flow

data which finally attempts to come to terms with the problem of poor coverage. New spherical harmonic analyses of world heat flow observations have appeared every few years since 1963 as the data set has grown, but they have all suffered from distortion caused by lack of measurements in certain critical areas (notably Africa). To overcome this difficulty in the most satisfactory way would require thousands of new measurements—which are simply not going to be made, at least within the foreseeable future.

Chapman and Pollack have therefore 'manufactured' the missing results using predictors based on known correlations: for continents, the observed correlation of heat flow with the age of the last tectono-thermal event, and for oceans, the observed relationship between heat flow and the age of the ocean floor. The resulting 12-degree spherical harmonic analysis (and its pictorial representation) is probably not far off that likely to arise from completely genuine data. It yields a world mean heat flow of 59 mW m⁻² (1.41 h.f.u.). □

The nucleolus on the Black Sea

from a Correspondent

The Fourth Nucleolar Workshop was held at Varna, Bulgaria, on September 14–19, sponsored by the European Cell Biology Organization and the Bulgarian Academy of Sciences. Further information can be obtained from the organiser, Professor A. A. Hadjiolov, Institute of Biochemistry, Sofia.

A MULTIDISCIPLINARY approach and a warm and friendly atmosphere contributed to make an exciting and successful meeting. During discussions of the nucleosome structure of chromatin P. Oudet (University of Strasbourg) reported results showing that they are 128 Å spheres of 194–200 nucleotide pairs and the four histones F2a₁, F2a₂, F2b and F3, while R. Tsanev (Institute of Biochemistry, Sofia) gave evidence for two internal supercoils of DNA per nucleosome wrapped around a protein core. Amongst the many questions yet to be elucidated is whether nucleosomes are present in actively transcribing chromatin. They have not been seen in active rRNA genes nor in lampbrush loops and they seem to characterise inactive chromatin.

Contributions on nucleolar fine structure brought about some modifi-

cations to the classical morphological model of the nucleolus. Evidence was presented by G. Goessens (University of Liege) that the fibrillar centres contain protein and some DNA, are labelled by ^3H -uridine pulses and are always associated with the dense fibrillar RNP components. In some cell types, the fibrillar centre can be followed during mitosis, it is also present in new-born nucleoli of heterokaryon cells (M. Bouteille, Paris).

Variation in nucleolar size and distribution of nucleolar components can reflect changes in activity. The formation of micronucleoli in ageing cells, characterised by degranulation and absence of condensed chromatin, seems related to cessation of RNA synthesis (K. Smetana, University of Prague). In contrast, small nucleolar fragments caused by α -amanitin retain the granular component and remain active in RNA synthesis (R. Simard, University of Montreal). The existence of a true nucleolus was demonstrated also in yeast cells by B. Stevens (University of Paris XI).

Evidence for a nucleolar compensation mechanism was given by V. Sakharov (Institute of Chemical Physics, Moscow) following ultraviolet microbeam irradiation of one of two interphase nucleoli. Cinematography showed an increase in volume of the unirradiated nucleolus simultaneous with a shrinkage of the damaged one.

The beautiful demonstration by the group of W. Franke (Cancer Research Centre, Heidelberg) of the fine anatomy of rRNA transcriptional units using the O. Miller spreading technique brought several new results. The start of the "Christmas Tree" may not correspond exactly to the initiation point since "prelude" transcripts are often seen and occasional long RNP fibrils may be interspersed in the initiation region, indicating that transcription and processing may go on together. The view that the primary transcript is larger than the first stable product and that the spacer is transcribed but immediately cleaved is supported by examples of long transcription units with almost no spacer and by 5-fluorouracil treatment where the spacer is eliminated and a larger than 40S RNA molecule is found in *Xenopus*. Regulation of transcription seems to depend on the rate of initiation. The concepts of heterogeneity and individuality of rRNA genes within one species and even within one nucleolus were subjects of some discussion.

Mapping of genes by restriction enzymes produced two important contributions. The five histone genes were shown by W. Schaffner (University of Zurich) to alternate with each other instead of being clustered in five separate groups. J. Engberg (University of

Copenhagen) demonstrated an unusual arrangement of the rRNA genes in *Tetrahymena* as a palindrome with two gene copies per intact 12×10^6 linear molecule.

Conservation of some rRNA sequences during evolution was demonstrated by S. Gerbi (Brown University) based on competition hybridisation and comparative fingerprint analysis and by M. S. Khan (University of Glasgow) and J. Klootwijk (Free University, Amsterdam) who found conservation occurred around methylation sites.

The three nuclear RNA polymerases can be manipulated and characterised by their different sensitivities to α -amanitin and heat inactivation. The yeast polymerase A studied by J. Retel (Free University, Amsterdam) is active in rRNA transcription *in vitro* with a preference for a high physical integrity of the DNA template and a low enzyme/DNA ratio. Using a technique of subnuclear fractionation J. Tata (National Institute for Medical Research, Mill Hill) emphasised the compartmentation of events in the nucleus. Polymerase B is found exclusively in the nuclear sap and euchromatin-rich fractions and most processing and polyadenylation occurs close to the site of synthesis.

Maturation of rRNA precursors may involve the simultaneous occurrence of more than one pathway (M. Dabeva, Institute of Biochemistry, Sofia) and the two rRNA species mature at different rates and sites. In *Chironomus*, the cytoplasmic distribution of newly-labelled 28S rRNA follows a gradient up to 6 days (U. Lönn, Karolinska Institute, Stockholm).

Research on extranucleolar sites of RNA synthesis and processing has received much impetus from collaborative efforts by J. P. Zalta (University of Toulouse) and E. Puvion and G. Moyne (Institut du Cancer, Villejuif) combining biochemical fractionation and electron microscopy. Newly-synthesised RNA can be located in perichromatin fibrils at the periphery of condensed chromatin; the labelling profile of this product corresponds to HnRNA.

A keyword of the meeting was post-transcriptional regulation. S. Penman (MIT, Boston) showed that growing cells process more of their pre-mRNA into cytoplasmic poly(A)-mRNA than do resting cells. Evidence was presented (K. Scherrer, I.B.M., Paris; E. Eghazi, Karolinska Institute, Stockholm; E. Serfling, Genetics Institute, Gatersleben) that pre-mRNA genes may be continuously transcribed in all cells but that control resides in the efficiency of processing into mature mRNA.

The next Nucleolar Workshop is planned for 1977 in Salamanca.

A most Friendly meeting

THE article regarding "a most Friendly meeting" (*Nature*, 257, 741; 1975) was flattering, but unfortunately misleading, in that it described as being completed experiments which have not yet been accomplished. Specifically, heterokaryons formed by the fusion of DMSO-induced tetraploid mouse erythroleukaemia cells with diploid human amniotic fibroblasts were said to have synthesised human globin. Unfortunately, these heterokaryons so far synthesise only mouse globin. It is possible that the authors were referring to experiments reported by one of us (Deisseroth A., *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, 72, 2682; 1975), in which continuously proliferating hybrid cells were generated by the fusion of human marrow erythroid cells with diploid mouse erythroleukaemia cells. These hybrids, when induced with DMSO, contain both human and mouse globin messenger RNA.

We do not want readers to think that human fibroblasts have been as yet induced to synthesise hemoglobin; these experiments are in progress.

BLANCHE ALTER
ALBERT DEISSEROTH

Astronomy from the Space Shuttle

from John Gribbin

A discussion meeting of the Royal Astronomical Society and the Institute of Physics—Uses of the Space Shuttle for Astronomy and Geophysics—was held on December 12 in London.

THE meeting highlighted the very different approach to scientific experiments in space necessitated by the advent of the shuttle. Previously, would-be experimenters have to some extent operated on the basis of envisaging a worthwhile experiment and then looking for a suitable way to get it off the ground; now, the American and European space agencies (NASA and ESA) are providing a standard launch vehicle and modular space laboratory, and it is up to the experimenters to make the best use of the facility provided.

J. Burger (ESA) set the scene with a background talk describing the work-

horse vehicle for low orbit work in the 1980s. The supposed prime reason for the development of the Shuttle is the economy of a re-usable facility. There has been some debate about just how economical the Shuttle is likely to be, but some scientific good may emerge from the political debate since in order to be cost effective the vehicle must certainly be flown as frequently as possible. The advantages of the Shuttle/Spacelab in providing a facility for repeated experiments, retrieval and repair of satellites and reducing the lead time from idea to orbiting experiment are in large measure counterbalanced by the need to work within a well-defined framework and the restriction of flights to between 7 and 30 days in orbit. Although Burger did not say so, the real situation from the scientists' point of view seems to be that this is the best Shuttle we've got, and with no other major launch facility on offer we had better make the most of it.

The basic facility is a Spacelab built up from 4 m diameter modules with a shirtsleeve environment and pallets open to space. Depending on the exact configuration, the mass carried is in the range 5,500-9,100 kg, with a floor loading of about 500 kg m² very similar to that in buildings; the volume of the closed modules may be between 5 and 8 m³, and the length of open pallet may be as little as 3 m or as much as 15 m. Up to four specialist experiment operators will be carried on Spacelab flights, and they will have available 4-5 kW of power with a peak short term facility of 9-10 kW and a total of 400-600 kW h at 28 ± 4 V. The Spacelab/Shuttle will not use solar cells, and depends on stored power. An onboard computer (64 k) will provide some data processing in orbit, and the stability of this space platform will give arc second pointing accuracy for payloads of around 2,000 kg.

Potential users of the facility seemed, on the evidence of this meeting, to divide between those (such as solar observers) who felt that 7-30 days in space is severely limiting and those (such as the rocket X-ray experimenters) who seemed almost overwhelmed at the prospect of such lengthy observations. This perhaps emphasises that Spacelab is unlikely to be ideal for anybody but will probably offer something for everybody. The infrared observers, represented by R. E. Jennings (University College, London), are among those to whom even 7 d observing is a long time (compared with balloon flights of a few hours) and plans are going ahead for two major telescopes, a 2-3 m instrument operating at ambient temperature with high spectral and spatial resolution, being studied by ESA, and a cryogenically cooled instrument with

high sensitivity but limited size, being studied by NASA. One major compromise in Jennings' eyes is the presence of people on the Shuttle and the need to test life support and other systems before venturing on long missions. He would like to see pallet-only missions lasting 30 days as soon as possible, without "people hopping about" inside the vehicle.

R. Griffiths (University of Leicester) pointed out that the high energy region of the spectrum cannot be studied from ground-based observatories at all, and that the observation time provided by Spacelab will be at least 10⁴ times more per flight than that provided by rockets. Simply flying tried and tested rocket type X-ray detectors on a Spacelab pallet is certain to produce an enormous increase in knowledge about the Universe at these wavelengths, and there is likely to be a Small Instrument Package System (SIPS) to provide mounting and accurate pointing for pairs of small experiments which can thus almost ride piggyback on a major Spacelab flight. But there is also the potential with Spacelab for a further quantum jump in observing techniques at X-ray frequencies, including development of a planned 60 cm telescope (3 m focal length) providing the facility for a variety of focal plane observations. Gamma ray astronomy seems at last to be producing something more than negative observations and upper limits, and will obviously benefit from the opportunities of the Shuttle; cosmic ray studies could move into new areas with searches for anti-nuclei and perhaps determination of isotope abundances, and one possibility for Spacelab is a complete high energy payload of X, gamma and cosmic ray equipment on one pallet.

This type of coordinated package also provides the major advantage of Spacelab to the solar physicist. A. H. Gabriel (Appleton Laboratory, Slough) stressed the need for coordinated observations of solar phenomena at a variety of wavelengths, giving a simultaneous picture of events at different temperatures and different heights in the solar atmosphere. The high resolution available above the atmosphere is equally important, and to round things off the first flight of Spacelab in 1980 should give time to have everything running smoothly in time for observations of the next solar maximum. But even 30 days is, of course, a short time for observations of many solar phenomena.

P. W. Hill (University of St Andrews) received the roughest ride, both in the limited question time and in private discussions afterwards, for his efforts to justify Spacelab as an observing platform in the visible part of the spectrum. It was generally

agreed that a 1 m telescope in orbit could produce results comparable to the 200 inch on the ground—but that it would probably cost at least as much as a new 200 inch. If the Shuttle was to be an optical astronomy facility alone, it would be a waste of time and money; an hour's observing at a major ground based observatory costs about £50, and Hill quoted an estimate that an hour's observing from the Shuttle will cost £50,000. Only in the far ultraviolet do the advantages of the Shuttle really become valuable for conventional-type optical astronomy, and an orbiting ultraviolet Schmidt camera to produce a sky survey at these wavelengths could be particularly valuable.

The value of the Shuttle for atmospheric, magnetospheric and plasma physics (the AMPS package) seems to be largely the opportunity to 'kick' the near-Earth environment and see what happens. L. Thomas (Appleton Laboratory) foresaw that by the 1980s we will have done the basic exploration of the magnetosphere through planned satellites, and will be entering the phase of testing hypotheses and looking for cause and effect relationships. Spacelab is certainly an appropriate platform for such experiments, and as well as the opportunity to study the magnetosphere by firing lasers, masers and electron streams into it, there is ample scope to monitor the upper atmosphere by observations across a chord of the tenuous upper layers providing long path lengths and giving data on low concentration constituents. Perhaps Spacelab will finally resolve the ozone/SST/freon.

Surprisingly, however, the most immediate and obvious astronomical benefits from Spacelab—and those which are certain to catch the public eye—will be the first photographs of the planets from a modest orbiting telescope. A 1 m telescope in orbit, said G. Hunt (Meteorological Office) will produce pictures of Uranus as good as the best ground-based pictures of Jupiter, pictures of Jupiter with resolution of 250 km or better equivalent to those from the Pioneer spacecraft, and comparably good pictures of the other planets. The particular significance of this is that the facility will provide many pictures over a period of years, whereas the spaceprobes provide only one-off series of snapshots. The advantages in the study of planetary atmospheres—the rapid rotation of Venus' atmosphere, the dust storms on Mars, the disturbances and spots on Jupiter—are obvious. But if the series of planetary space probes from NASA are remembered by most people simply for their photographs, it seems inevitable that the public image of Spacelab will be a platform for viewing the planets. □

review article

Quantitative variation and gene number

James N. Thompson, Jr*

Gene number estimates from fine structure analysis seem to be incompatible with the number of genes often presumed to affect quantitative characters. This paradox can be resolved by reconsidering the available evidence about the nature of polygenes.

MUCH of the discussion about the number of genes in eukaryotic genomes has centred on the discrepancy between the probable gene number, based on mutation saturation rates and fine structure analyses, and the apparent coding potential of the DNA¹⁻⁵. The haploid *Drosophila* genome, for example, contains enough DNA to code for over 100,000 genes. Recent studies, however, tend to support the idea that the actual number of genes is only about 5,000, approaching a 1 : 1 correspondence between functional units and bands or interbands in polytene chromosomes. Thus, the actual number of genes appears to be 1 or 2 orders of magnitude less than the number which could be coded for by the available DNA. Such a relationship clearly has important implications for chromosome organisation and for the control of development^{6,7}.

But quantitative genetics often seems to make quite different assumptions about gene number. Many characters, such as human height or tail length in mice, are considered "polygenic"; that is, genes which affect them are believed usually to have effects that are small relative to those of other sources of variation. A normal phenotypic distribution for such a character is often interpreted as showing the segregation of a large number of genes. In addition, the fact that the expression of hundreds of mutant alleles can be modified by selection is believed to indicate that these selection responses are often due to changes in the linkage of a large number of polygenes.

This seems to present a paradox. Deletion analyses and studies of lethal complementation groups suggest that there is a comparatively limited number of genes affecting development, while studies of quantitative characters seem to uncover a vast number of genes having morphological effects.

But this paradox is really traceable to basic misunderstandings about the nature of polygenic variation. "Polygene" is a nonspecific term used to describe what is probably a very heterogeneous set of gene effects. It was originally defined in the context of biometrical analysis⁸. One of the early, and quite necessary, assumptions which biometrical models made about genetic systems was that quantitative variation was produced by many genes having small, similar effects. Unfortunately, some have since come to think of this description as an established fact rather than as a simplifying assumption.

It is appropriate, therefore, to ask whether one must necessarily conclude from a normal phenotypic distribution that a given character is affected by the segregation of a

large number of polygenes. The answer is "no", a large variance can be caused by the segregation of only a few genes just as it can be caused by the segregation of a large number. The theoretical distribution which underlies the sample shown in Fig. 1a is produced by the segregation of only three genes. One of these has three times, and one has twice, the effect of the third gene. The distribution approaches a normal distribution, even though only two loci account for more than 90% of the variance. When the effects of environmental variation are taken into account (Fig. 1b) even segregation at a single locus can produce a continuous distribution of phenotypes. Clearly, there is no justification for automatically assuming that a continuous distribution reflects the segregation of a large number of polygenes.

Although experimental analysis of quantitative characters is usually more complex, some bristle and venation characters in *Drosophila melanogaster* have confirmed that a relatively small number of genes can account for the observed variance and response to selection.

Many major mutants affect the formation of wing veins

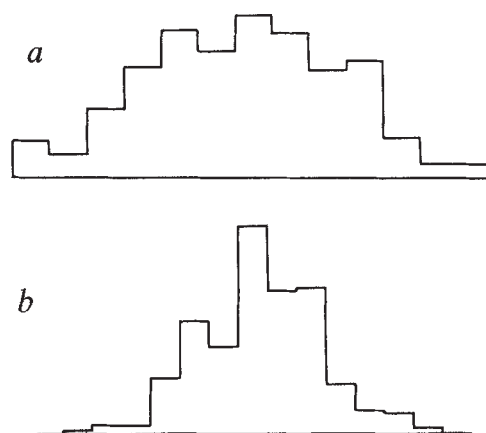


Fig. 1 *a*, Random samples of 150 individuals taken from a theoretical distribution of only three segregating loci. The effects of the environment have not been considered in this example. Superimposing environmental variation on this distribution of phenotypes, as is typical of quantitative characters, would blur phenotypic classes even more. *b*, Random sample from an F_2 in which only one locus is segregating. The effects of environmental variation have blurred the classes to produce a more nearly continuous phenotypic distribution. In this example, heritability is about 50%. Clearly, there is no justification for automatically assuming that a continuous distribution reflects the segregation of a large number of polygenes.

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in *Drosophila*. Not surprisingly, the expression of vein length in these mutants is, in turn, affected by polygenes, and vein length responds readily to artificial selection. Studies of these vein length polygenes show several things. One striking observation is that all tested natural populations seem to be polymorphic for polygenes affecting vein length—all samples of wild populations are segregating for alleles which either lengthen or shorten the veins of mutant

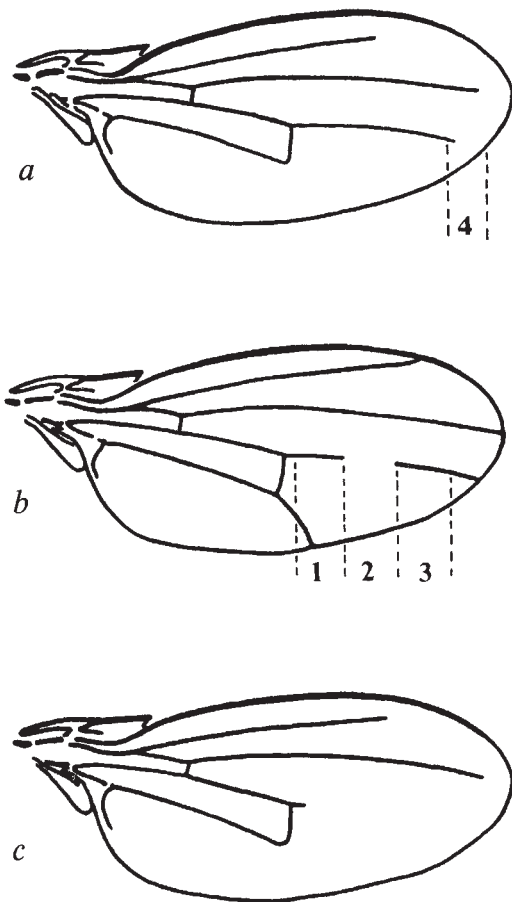


Fig. 2 Wings of *Drosophila melanogaster*, showing regions of the fourth longitudinal (L4) vein affected by individual polygenes. The difference in expression of *ve Long* (a) and *ve Short* (c) selection lines can be accounted for by only two or three loci. One of these (located on the right arm of chromosome II, ref. 23) eliminated vein from the middle of the L4 vein (b, region 2). The other polygenic loci influence the expression of the second chromosome polygene by enlarging the gap formed in the middle of the L4 (regions 1 and 3). The same gene or genes may act on both regions 1 and 3. The *ve* mutant itself does not form vein at the tip (region 4), even in Long selection lines. Thus, the *ve Short* phenotype (c) is simply the summation of the background effects (mid-vein) and the *ve* phenotype (distal-vein), leaving the base of the L4, which is not affected by either set of genes, intact.

flies by a small amount, although these polygenes do not have any recognisable phenotype in the wild flies in which they are found.

One might interpret this observation as showing that a large number of polygenes affecting vein formation exist in nature. On closer examination we find that this is not necessarily true. By locating regions in a chromosome which have an effect on the character, one can isolate individual polygenic loci⁹. Even though a polygenic locus could be a complex of two or more closely-linked loci, this technique still gives us a reasonably clear idea of the number of factors which account for the majority of the genetic variance. In the case of polygenes affecting vein length in

the mutant *veinlet* (Fig. 2), only two or perhaps three loci accounted for most of the difference between a line selected for longer and one selected for shorter L4 veins.

The same general conclusion emerged from studies of polygenes affecting sternopleural bristle number in *Drosophila melanogaster*. Thoday and his colleagues¹⁰⁻¹³ found that only five loci accounted for more than 85% of the difference between a high line having a mean of about 40 bristles a fly and a control line having about 20 bristles a fly. (Biometrical, rather than genetic, estimates suggest that approximately 100 polygenes affect a similar character—abdominal bristle number¹⁴.)

In this context, however, it must be remembered that one can only study genes affecting the variance of a character in a population sample. Although a given polygene can appear in more than one population^{15,16}, each population may carry its own polygenic combinations. There may also be polygenes which have extremely small effects. These can not generally be examined by standard polygene location techniques, but by the same token they will have only limited importance for the determination of the variance. Many of these may be pleiotropic effects of other loci, though there is little evidence on this point. In addition, there may be undetected homozygous alleles in the sample being measured, since one can only detect segregations contributing to the variance. All of these situations will increase the total estimate of polygene number. But even with these reservations, there is no compelling reason to think that the total number of loci affecting any particular quantitative character is exceptionally large.

The evidence that a relatively small number of genes can, in principle, account for the genetic variation of various characters solves only part of the problem. How does one reconcile the fact that the sum of all polygenes affecting all characters still produces an estimate which is inconsistent with those of Judd^{1,3}, Hochman², and others? In addition, is it possible to explain complex characters, such as body weight, in which it appears that large numbers of polygenes are actually involved? These two questions can best be answered by considering the possible functions of individual polygenic loci.

Again, venation and bristle characters in *Drosophila* are model systems. If the polygenes affecting these characters are specific modifiers of a particular locus (suppressors or enhancers affecting the control of the mutant locus—see, for example, ref. 17) the total number of polygenes in nature must be much larger than the number of mutants having morphological effects. If, on the other hand, polygenes influence some step in a particular developmental process and only indirectly modify the expression of a mutation in the same process, the minimum polygene estimate can be greatly reduced because one polygene could contribute to the variation in a number of different mutant phenotypes. In the first situation, polygenes might be a special class of control genes; in the second situation, they may simply be minor variations at familiar loci. Although a few mutant-specific polygenes have been reported¹⁸⁻²⁰, recent studies of the modifiers of vein mutants support the second hypothesis, that, in general, polygenes seem to act primarily on some developmental step and only indirectly modify mutant expression²¹⁻²³.

Some of the evidence for this conclusion is summarised in Table 1. Six different vein mutants²⁴ were selected for longer or for shorter vein length (*veinlet*, *radius incompletus*, *short vein* and *cubitus interruptus*) or for extra vein fragments (*plexus* and *net*). Selection accumulates in each line the polygenes which have the relevant effect on the phenotype. By comparing the contribution of individual chromosomes with a standard the overall phenotypic effects of the polygenes on each chromosome can be measured. Recombination studies of some of these lines show that the majority of the effect of each chromosome is due to only

Table 1 Summary of chromosome substitutions of selected polygenes from one mutant background in to another

Vein affected by polygenes	Chromosome substituted*	First mutant (source)†	Second mutant	Effect in first mutant	Effect in second mutant
2	II	<i>ve</i> L-2	<i>ri</i>	+	+
2	II	<i>ve</i> L-3	<i>ri</i>	+	+
2	II	<i>ve</i> S-2	<i>ri</i>	+	+
2	II	<i>ri</i> L	<i>ve</i>	+	+
2	III	<i>tg</i> ^C S	<i>shv</i>	—	—
3	II	<i>ri</i> L	<i>ve</i>	None	None
3	II	<i>ri</i> S	<i>ve</i>	None	None
4	II	<i>ve</i> S-2	<i>ci</i>	—	—
4	II	<i>ve</i> S-4	<i>ci</i>	—	—
4	II	<i>ri</i> L	<i>ci</i>	None	+
4	II	<i>ri</i> L	<i>ve</i>	None	None
4	III	<i>shv</i> L	<i>ci</i>	+	+
4	III	<i>shv</i> S	<i>ci</i>	—	—
4	III	<i>ve</i> S-4	<i>ci</i>	—	—
4	III	<i>ve</i> S-4	<i>shv</i>	—	—
4	III	<i>px</i> L	<i>shv</i>	+	+
4	III	<i>px</i> S	<i>shv</i>	—	—
4	III	<i>net</i> L	<i>shv</i>	+	+
4	III	<i>net</i> S	<i>shv</i>	—	—

These substitutions confirm that at least a proportion of the polygenes affecting vein length have the same influence on the phenotypes of non-homologous mutants.†

* Chromosome extracted from the selection line (without carrying a venation mutant) and substituted in to a control line of the second mutant.

† The original selection line. L, longer veins or extra vein fragments; S, shorter veins or fewer vein fragments; numbers refer to tests of parallel selection lines of the same mutant.

‡ Mutant symbols: *tg*^C (telegraph of Carlson) and *ri* (*radius incompletus*) shorten vein 2; *ci* (*cubitus interruptus*) shortens vein 4; *ve* (*veinlet*) and *shv* (*short vein*) shorten all veins; *net* (*net*) and *px* (*plexus*) have fragments of extra venation.

one or two loci. This is consistent with the earlier studies on polygene number.

The next step is to test the specificity of the polygenes on each chromosome. Using marked balancers, chromosomes from one mutant line can be substituted for a control chromosome in the genome of a different vein mutant. It is clear from the substitutions summarised in Table 1 that the vein length polygenes on these chromosomes act in a general way on vein formation insofar as they have qualitatively similar effects upon the phenotypes of different mutants. Those which lengthen the second longitudinal vein in *veinlet*, for example, also lengthen the second longitudinal vein in *radius incompletus*. Those which decrease the amount of extra venation in *plexus* also decrease the amount of vein in *short vein*. With one minor exception, all of these substitutions support the hypothesis that a proportion of the polygenes are acting in a general way on some aspect of vein formation and only indirectly influencing the expression of related mutations. This, therefore, reduces the number of polygenic loci which one must postulate to explain the vast amounts of variations observed in mutant expression. I am confident that many such examples could be found.

Another point which emerged from the analysis of vein expression polygenes was that they were often specific to one region of the wing. One polygenic locus would affect the formation of one vein only. This was most clearly shown by selection in *veinlet*, in which all of the longitudinal veins are normally shortened (Fig. 3). One vein could be selected to be shorter, while another vein was selected to be longer. This vein-specific action was also reflected in the contributions of different chromosomes. Chromosome II might carry polygenes affecting one vein while chromosome III would carry polygenes affecting another. This is consistent with the concept of individual polygenes acting on components of some developmental process. Polygenes acting specifically on the *veinlet* locus would have been expected to modify all veins in the same way.

But the demonstration of 'region-specific' effects makes an even more important contribution to the problem of polygene function and number. It underlines the fact that one is really concerned with a phenotype which is a compo-

site of many separate, though integrated, processes. The longitudinal veins in the *Drosophila* wing are, to a degree, genetically and developmentally independent. This is seen in the fact that some mutations affect only one vein²⁴. Thus, in considering polygene number, one should really be concerned with the complexity of the phenotype—the number of component processes which polygenes could influence.

This point can be illustrated further by the sternopleural bristle system. Spickett²⁵ isolated three polygenic loci which accounted for the majority of the differences between a selected high bristle number line and a control stock. He

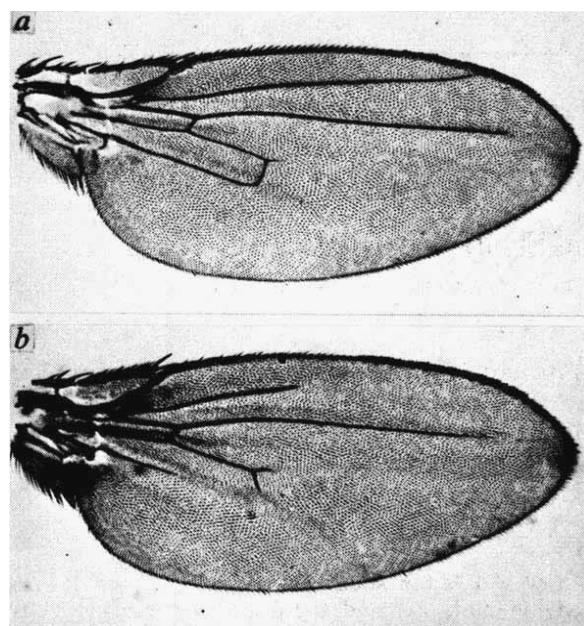


Fig. 3 Representative phenotypes from two *veinlet* selection lines, in which vein lengths were modified in different directions. *a*, The L4 was selected shorter, and the others were selected to be long. *b*, The L3 was selected to be long, and the others were shortened. Vein-specific polygenic effects were responsible for the responses to selection²³.

then looked at the developmental effects of each of these loci. One caused a general increase in cell number, indirectly increasing the number of bristle-forming cells. One increased the distribution of small bristles on the ventral part of the sternopleurum. The third locus altered the timing of cell division of one large dorsal bristle, causing two bristles to be formed in its place. Each of these loci affected a different developmental process, but directly or indirectly contributed to the variation in sternopleural bristle number. A similar situation was described by Jacobson²⁶ who found that selection for increased facial vibrissae number in mice was caused by the development of extra hair follicles, whereas back selection affected not the number of follicles but the suppression of their formation of hairs. Variation in two quite different processes was involved. In a complex character, such as body weight, one could imagine the number of contributing processes to be even larger.

The more 'complex' a character, the more polygenes one should expect to influence it. Unfortunately, such a criterion is based more on intuition than on judgement. But the expectation is, I believe, logical, and it is consistent with our limited knowledge of polygene function.

Even though polygenic effects are numerous, the actual number of loci is probably of the same order as the number of 'major mutant' loci. If it is true that polygenic loci and major gene loci are essentially the same, this implies that many polygenes are isoalleles of known major mutants. Certain technical problems²⁷ make this difficult to test in most cases, but examples of isoalleles contributing to quantitative variation are known^{16,28}. In a sense, then, polygenes may be the normal wild-type alleles.

We still have much to learn about the variety of gene effects which may be classed under the term 'polygenic variation'. In spite of this, there seems to be no compelling

reason to believe that the total number of loci affecting quantitative characters is incompatible with estimates of gene number based on major mutant complementation studies.

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articles

Metamorphic belt and volcanic arc migration in New Zealand

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The Phanerozoic development of New Zealand can be viewed in terms of four eastward migrating cycles of closely similar character, all of which are related to westward dipping Benioff zones. Paired metamorphic belts are produced during the final stages of each cycle.

It is now well established that the Cretaceous Rangitata Orogeny in New Zealand produced two parallel belts of contrasting metamorphism: a western belt characterised by granites, and an eastern, low temperature, higher-pressure belt characterised by wide zones of low grade metamorphism¹. According to plate tectonic theory this pairing would have developed over a westward dipping Benioff zone. Similarly, the Permian, Cretaceous, Tertiary

and Recent volcanic arc structures in New Zealand have been related to westward dipping Benioff zones^{2,3}.

I show here that it is possible to view the entire Phanerozoic development of New Zealand in terms of westward dipping Benioff zones, a view attractive for its simplicity. Four closely similar cycles can be recognised, each ending in an orogeny producing a paired metamorphic belt. Each new cycle is developed to the east of the preceding one.

Sedimentary belt migration

Three belts of more or less fossiliferous sediments can be recognised. The eastern margin of each belt in turn becomes the western margin of the younger belt so that they become progressively younger to the east (Fig. 1). The oldest (western) belt (equal to the central belt and

eastern part of Cooper's western sedimentary belt⁴) contains Cambrian to Upper Ordovician sediments; the central belt (the eastern sedimentary belt of Cooper⁴) contains uppermost Ordovician to Devonian sediments; and the eastern belt (Hokonui facies) contains Permian to Jurassic sediments. The Hokonui facies was derived from a land-mass to the west³, and was probably laid down on oceanic crust now represented by an ophiolite complex⁶.

The source area and nature of the basement for the two older belts have not been established; there is no evidence of a continental basement. In each case, older rocks lie to the west and form a possible source, whereas there is no known source to the east.

Two other sedimentary belts of quite different character can be recognised. First, covering large areas to the west of the oldest fossiliferous belt is a monotonous sequence of sparsely fossiliferous Cambrian to Ordovician^{7,8} turbidites (Fig. 1) which were laid down on a Precambrian continental crust⁹. Their character and subsequent metamorphic history differs from that of the similarly aged sediments in the oldest fossiliferous belt. Second, to the east of the youngest fossiliferous belt is a widespread and thick succession of sparsely fossiliferous sediments, mainly of Carboniferous to Jurassic age (Torlesse Supergroup) (Fig. 1). Unlike the similarly aged Hokonui facies, these sediments were derived from the east⁹, and may be completely allochthonous, possibly having been conveyed from the east by seafloor spreading and welded to the Hokonui Belt during the Cretaceous Orogeny (ref. 10 and J. D. Bradshaw personal communication).

Non-orogenic volcanic arc migration

Several volcanic arcs have been recognised in New Zealand. A distinction can be made between non-orogenic arcs and those associated with orogeny and granite formation.

The non-orogenic volcanic arcs migrate progressively eastwards with time (Fig. 2). The oldest andesitic arc of Cambrian age borders the east side of the oldest fossiliferous sedimentary belt⁴. Volcanics interlayered with sediments of Ordovician–Silurian age occur on the east side of the central sedimentary belt¹¹, but their character is obscure since they are invaded by Cretaceous granite; the massive Rotorua Complex (Fig. 2) of basic igneous rocks lies in this belt, but it too is metamorphosed by Cretaceous granite, and ages as various as Cambrian to Cretaceous have been suggested. Late Carboniferous diorite and more basic intrusions also occur on the east side of the central belt^{11,12}, again in direct line with the Rotorua Complex. Further east, Permian volcanic arcs—one has been interpreted⁶ as an ophiolite complex (see Fig. 2)—were developed in association with sediments of the Hokonui facies. Finally, between the Rangitata and the present Kaikoura orogeny, a late Cretaceous volcanic arc developed in the exotic Torlesse Belt³.

The oldest four arcs are associated with and parallel to the fossiliferous sedimentary belts of the same age. It may be speculated that if the allochthonous Torlesse Supergroup had not piled up against the Hokonui facies, the Cretaceous arc would similarly have been associated with a belt of late Cretaceous fossiliferous sediments laid down to the east of the autochthonous New Zealand land mass.

The chemical variation in the Cretaceous and Permian arcs has been ascribed to a westward dipping Benioff zone³.

Metamorphic belt migration

Four main periods of folding or orogeny can be recognised in New Zealand: a Miocene–present day Kaikoura Orogeny; a late Jurassic–mid Cretaceous Rangitata Orogeny; a Devonian–Carboniferous Tuhua Orogeny; and

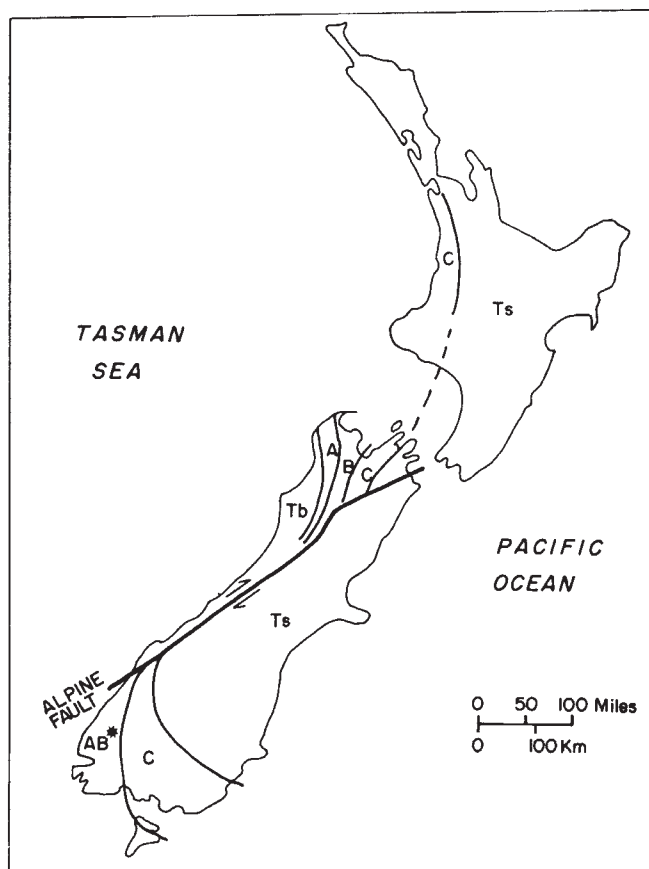


Fig. 1 *a*, *b* and *c*, Belts of more or less fossiliferous sedimentary rocks: *a*, Cambrian–Upper Ordovician; *b*, Upper Ordovician–Devonian; *c*, Permian–Jurassic. *Tb*, Area of Cambrian–Ordovician turbidites; *Ts*, rocks of the allochthonous Torlesse Supergroup. *ab**, belt which, because of intense Cretaceous metamorphism, cannot be differentiated in Fiordland.

a late Ordovician period of folding and granite intrusion (as yet unnamed).

As with the modern Mizuho Orogeny of Japan, the metamorphic effects of the Kaikoura Orogeny are difficult to judge. The opening of the Tasman Sea in late Cretaceous times¹³, the development of the important transcurrent Alpine Fault, and the general fragmentation of Gondwanaland since the Mesozoic, have created what is probably an atypical asymmetry in the effects of the Kaikoura Orogeny. A Benioff zone dips westwards only under the North Island, and the submarine trench off the east coast similarly dies out southwards. It is reasonable to suppose that a paired metamorphic belt is developing under the North Island, as in present day Japan¹⁴. The high temperature belt is represented by successive volcanic arcs from the Miocene to the present (developed mainly where sediments of the Torlesse Supergroup crop out) which produced granites and voluminous ignimbrites, which are presumably the superficial expression of granite. According to plate tectonic theory, the trench zone represents a developing area of high pressure metamorphism. The median boundary of the paired belts must lie parallel to the eastern coast of the North Island, to the east of the ignimbrite belt (Fig. 3).

The median boundary for the Rangitata Orogeny is well documented¹, and lies between the old New Zealand land-mass and the post-Tuhua, Hokonui facies sediments (Fig. 3). The paired belt with granites to the west of the median boundary indicates subduction from the east.

Median boundaries for the Tuhua and pre-Tuhua Orogenies have not previously been proposed, but there is

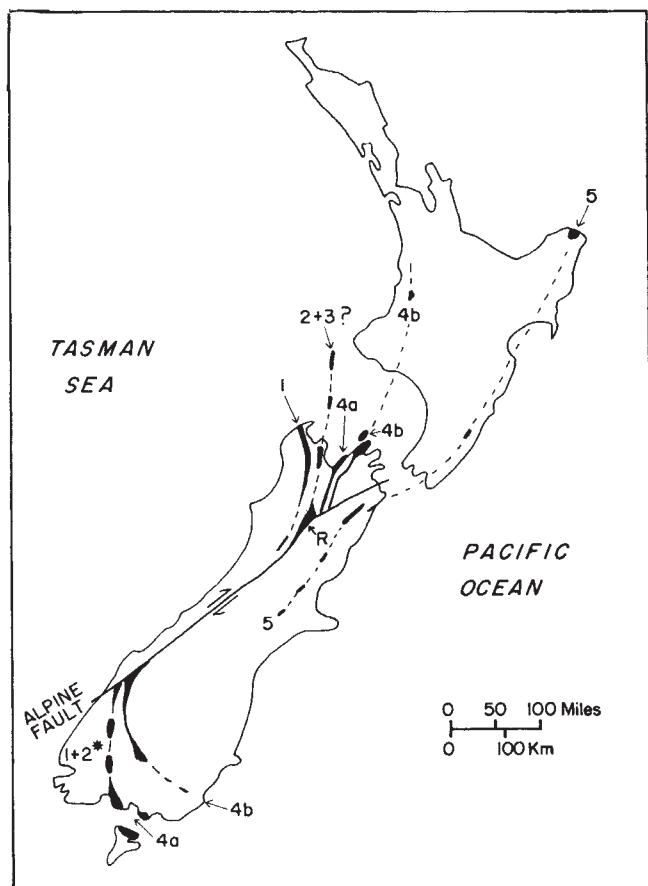


Fig. 2 Non-orogenic volcanic arcs: 1, Cambrian; 2, Upper Ordovician-Silurian; 3, Carboniferous; 4, Permo-Trias (4b may be Permian ophiolite⁹); 5, Upper Cretaceous. Fiordland geology; (1+2*), not yet elucidated. Extension of 2+3 offshore based on drillhole data²⁴. R, Rotoroa Complex.

good evidence that older boundaries do exist to the west of, and parallel to, the younger boundaries.

Granites of Tuhuan age abound in the west and north-west of the South Island¹⁵, and produce andalusite-sillimanite hornfels at their contacts. The most easterly Tuhuan granites intrude the Cambrian-Ordovician sediments of the western belt. Tuhuan deformation of the Upper Ordovician-Devonian sediments in the central sedimentary belt (Fig. 1) was accompanied by the formation of wide zones of low to moderate grade schists (up to staurolite grade) which commonly contain almandine. Although the formation of the schists has been regarded as resulting from^{16,17} the intrusion of Cretaceous granites, that view is erroneous. Andalusite and sillimanite have formed at the immediate contacts of the granites, and the thermal effect has been superimposed on already existing almandine schists; if the low pressure Cretaceous metamorphism had produced the schists, andalusite should appear in the assemblages before almandine, which it does not. The superimposition of the thermal effect is supported by textural studies near Springs Junction (S. W. Quek, personal communication). The voluminous uppermost Ordovician marbles contain abundant biaxial calcite (N. Newman, personal communication) which has been interpreted¹⁸ as a possible indication of inversion from high pressure aragonite. The general aspect in this belt is, therefore, of moderate to high pressure metamorphism. In common with modern orogenic belts, it seems that a Tuhuan median boundary separating granite activity from a belt of higher pressure metamorphism can be located close to the junction of the old landmass and the pre-orogenic sedimentary belt (Fig. 3).

The pre-Tuhua Orogeny is even less well documented than the Tuhuan, but recent radiometric work provides good evidence for the position of a median belt. The slaty cleavage formation in the sparsely fossiliferous Cambrian-Ordovician sediments (Fig. 1) is now dated at 440 Myr (ref. 19) which place it in the Upper Ordovician²⁰, and which precisely correlates with the migration of sedimentary deposition from the western to the central belts. The slaty cleavage was induced by the gravity sliding of wet (or dehydrating?) sediments in association with granite intrusion and a high geothermal gradient^{21,22}. Granites older than Tuhuan (about 450 Myr old) occur along the western coast of South Island (C. J. D. Adams, personal communication); their eastern limit is unknown, but is viewed here as coinciding with the eastern limit of the common structural pattern in the cleaved sediments. The fossiliferous sediments of the western belt (Fig. 1) underwent a complex deformation before the formation of the central belt. This deformation has generally been ascribed to the Tuhuan Orogeny¹¹, but in view of the absence of the early phase of folding in the sediments of the central belt, I regard the deformation as Ordovician (the only evidence that the deformation is Tuhuan is the presence of supposed but undated uppermost Ordovician marble slivers along the base of some thrusts¹¹). Large areas of low grade schist with abundant stilpnomelane in some assemblages²³ indicate a relatively high pressure metamorphism. The pre-Tuhuan median boundary separating granites on the west and higher pressure metamorphics on the east can therefore be drawn to the west of the Tuhuan median boundary (Fig. 3).

The metamorphic belts for both Palaeozoic orogenies

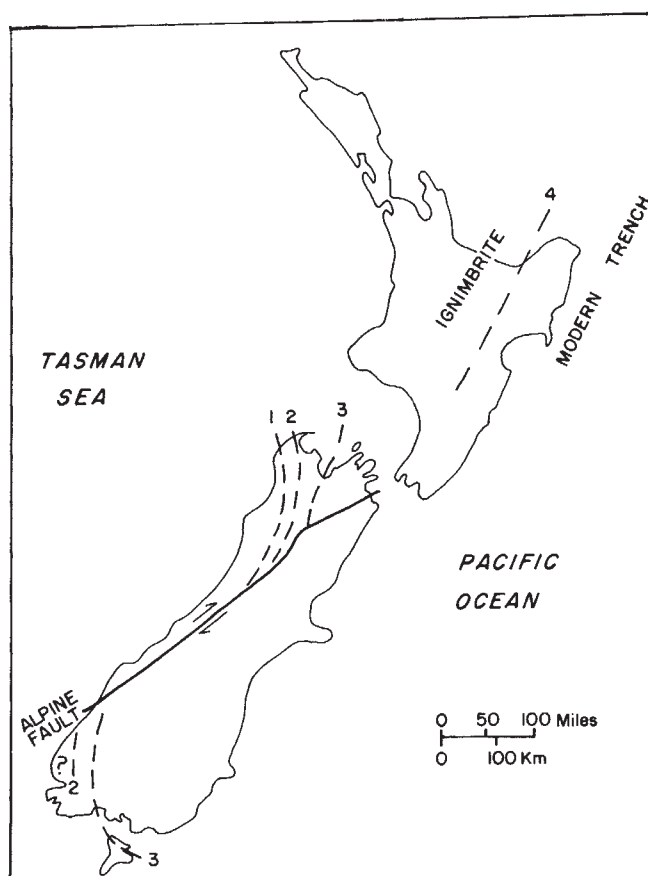


Fig. 3 Median boundaries of paired metamorphic belts, separating low pressure granite activity on the west from higher pressure metamorphism (without granites) on the east: 1, pre-Tuhuan Orogeny; 2, Tuhua Orogeny; 3, Rangitata Orogeny; 4, Kaikoura Orogeny.

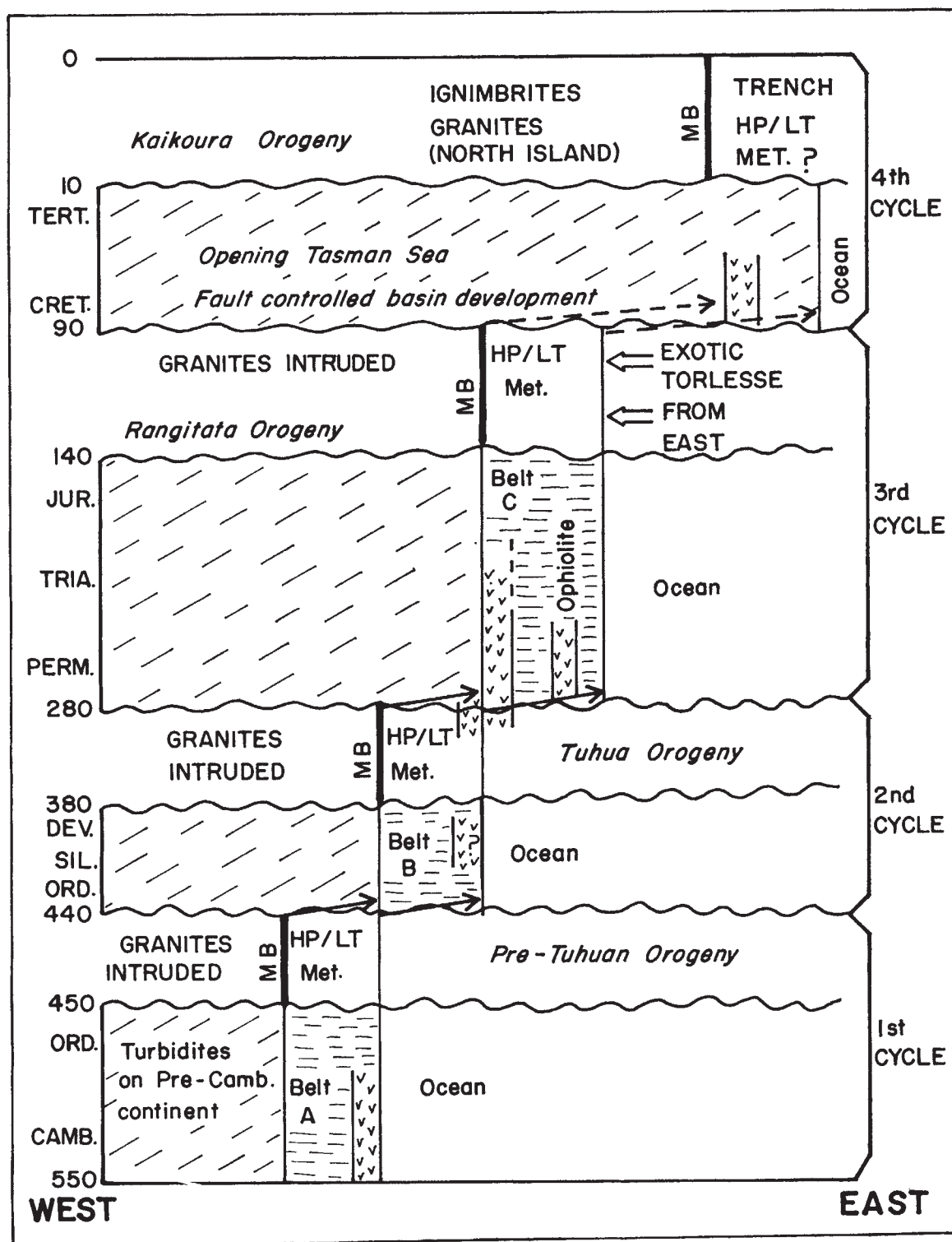


Fig. 4 Diagram summarising in time and space the cycles of sedimentation, non-orogenic volcanic arc formation and paired metamorphic belt formation. MB, Median boundary of the paired metamorphic belts. Belts A, B and C as in Fig. 1; V, non-orogenic volcanic arcs.

run parallel to the Rangitatan and modern trends, and the pairing with granites to the west and higher pressure metamorphics to the east, consistently indicates a westward dipping Benioff zone.

Eastward migrating cycles ending in orogeny

It is possible (see Fig. 4) to recognise a common cycle of events, repeated successively at more easterly locations. It seems that the entire Phanerozoic history of New Zealand

can be related to a series of Benioff zones dipping westwards under the New Zealand landmass. Each cycle involves three stages. First, deposition of a fossiliferous sedimentary belt directly to the east of a continental landmass (except for the latest cycle, which has been interfered with by the introduction of the allochthonous Torlesse Supergroup, and the fragmentation of Gondwanaland); second, a pre-orogenic volcanic arc or arcs associated with the sedimentary belt; and third, an orogeny producing

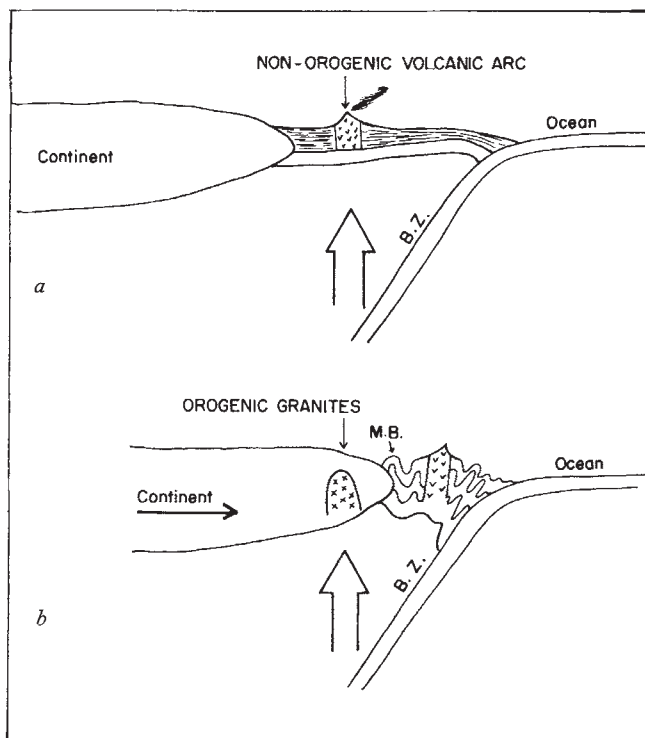


Fig. 5 Possible explanation for the relative positions of the non-orogenic volcanic arcs and orogenic granite belts. See text for discussion. BZ, Benioff zone; MB, median boundary of the paired metamorphic belts.

a paired metamorphic belt, the median boundary lying between the old landmass and the sedimentary belt.

The fossiliferous sedimentary belts probably all have a basement of oceanic crust which may be represented in at least one belt by an ophiolite complex⁶. Each new cycle represents the initiation of a new Benioff zone parallel to, but east of, the previous zone.

An important implication is that an ocean, like the Pacific, has existed to the east of New Zealand since at

least the Cambrian. Japan shows a similar parallel development of paired metamorphic belts from the Palaeozoic to the present¹⁴.

Relationship of volcanic arcs to metamorphic belts

The standard picture of a Pacific orogenic belt, as deduced, for example, from Japan¹⁴, is that of a volcanic arc coinciding with the granitic metamorphic belt of high temperature/low pressure. In New Zealand, it seems that between orogenies the arc lay in the pre-orogenic sedimentary belt destined to suffer a high pressure metamorphism (Fig. 4); during the ensuing orogeny, the arc becomes transposed relatively to the west to lie under the old landmass. An explanation may be that an accelerated spreading rate during orogeny causes the landmass to compress and override the sedimentary rocks to the east, so that the high temperature effects are apparently transposed westwards (Fig. 5); in other words, it may be the continent that moves rather than the zone of high heat flow.

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Summer phytoplankton blooms and red tides along tidal fronts in the approaches to the English Channel

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Tidal mixing on the continental shelf in the South-western Approaches to the English Channel produces a situation in the summer where within a few miles the warmer surface water becomes completely mixed with the underlying colder water. These frontal boundaries may be followed for 100 nautical miles. By combining physical and biological oceanographic disciplines it has become clear that these boundaries are the sites of phytoplankton blooms which, in favourable conditions, may develop into red tides.

OUR understanding of primary production in the sea seems to be hampered by an inadequate knowledge of the turbulent nature of the physical environment in which the phytoplankton exist. Progress in quantifying the distribution and growth rates of marine phytoplankton has taken two main directions; that of modelling¹⁻³, and the more statistical approach relating the variance and coherence of temperature and chlorophyll *a* to horizontal space scales⁴⁻⁶. Useful as the modelling approach has been in general, its main weakness is the uncertainty involved in specifying the mixing coefficients, which for the shelf region in the Western Approaches to the English

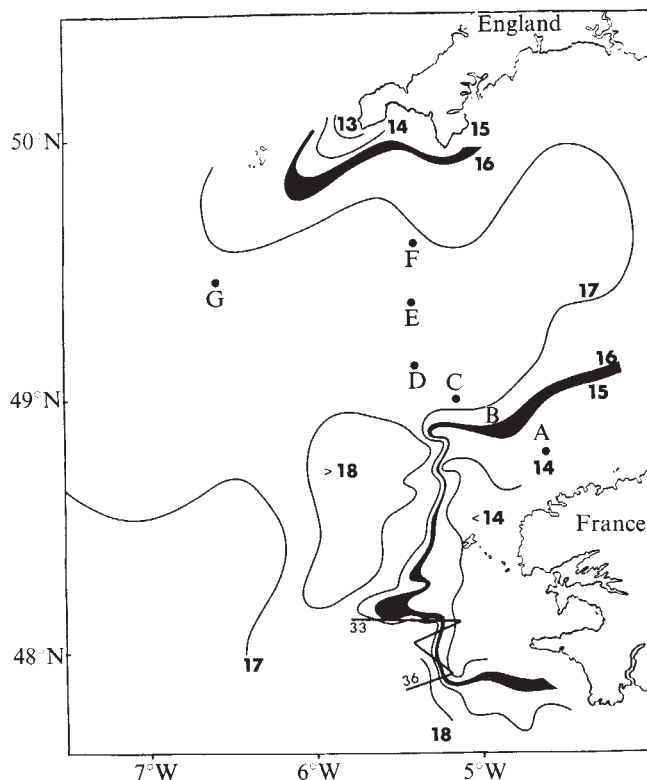


Fig. 1 Sea surface temperature contours obtained between July 26 and July 30, 1975. The 15–16 °C contours define the shelf front or the outcropping of the seasonal thermocline at the sea surface.

Channel may vary from 1 to $10^7 \text{ cm}^2 \text{ s}^{-1}$ (refs 7 and 8). The statistical approach is also a potentially valuable technique, but mechanisms of mixing which may be important in determining the distribution of phytoplankton are easily obscured. Here we show that the distribution and production rates of phytoplankton in the approaches to the English Channel are fundamentally linked with the turbulent nature of the environment. At this early stage a simple description of the possible operative mechanisms involved may be more illuminating and basic to our understanding than any attempt to define diffusion parameters or the statistical properties of the environment.

Surface temperature and chlorophyll *a*

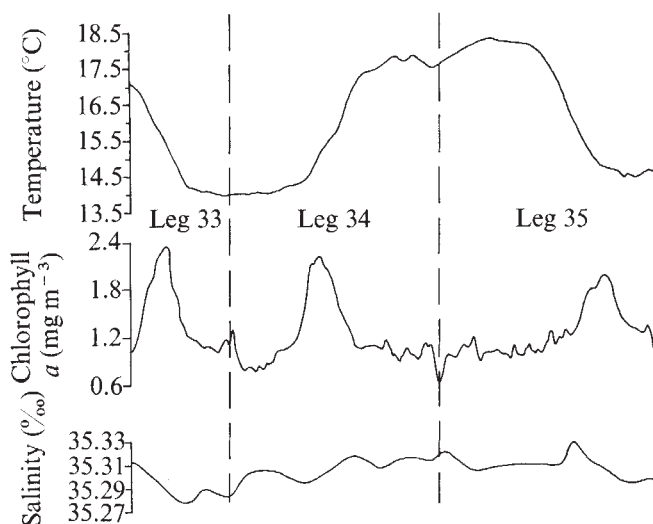
It is surprising that for an area like the English Channel, where hydrographic data have been collected for nearly a century, and for which mean monthly temperature distributions were available in atlas form 40 yr ago⁹, large surface temperature discontinuities that may be followed for up to 100 nautical miles (Fig. 1) have not been described. These structures mark the transition between well stratified shelf water and well mixed coastal water, or the outcropping of the thermocline at the sea surface. Stratification nearer the shore is also often evident, caused predominantly by warm, less saline water running off the land. Around the coast of France the transition can be defined by the maximum surface temperature gradient and in July 1975 was represented by the 15–16 °C surface temperature contours. In June 1974, it was similarly positioned and readily identifiable between the 13 and 14 °C contours¹⁰. With hindsight this feature can be recognised in earlier work¹¹, but it is, of course, more recent techniques, giving continuous records of hydrographic data, that have helped to define these regions clearly, and allow unambiguous interpretations that are often not possible with the more traditional methods based on sampling at discrete stations. Examples of continuous traces of temperature, salinity¹² and chlorophyll *a*¹³ can be seen in Fig. 2. These traces were obtained south of Ushant (legs 33, 34 and 35, see Fig. 1) where the frontal characteristics

were particularly well developed^{14–16}. Here about 50 % (~ 1.5 °C) of the temperature change across the region occurred within 3,000 m. Patches of floating weed were often evident in this zone¹⁷ and also conspicuous was the frequent presence of fog over the cooler water. The lack of any large salinity changes (< 0.03 ‰) or consistent correlations of salinity and temperature suggest that advection of warm surface water may not in general be responsible for the summer surface temperature distribution in the Western Approaches although historically the role of advection, rather than local surface heating and vertical mixing (over horizontal scales of the order of the tidal excursion), has been assumed dominant¹⁸. The chlorophyll *a* trace does, however, show a clear association with the temperature gradient. It is this association between temperature anomalies and phytoplankton distribution within the general framework of the presented hydrography, and the special circumstances that may bring about summer plankton blooms, and even red tides, which we are particularly concerned with here.

Vertical structures of temperature and chlorophyll *a* through a region of phytoplankton bloom

The vertical profiles of temperature and chlorophyll *a* were more conveniently investigated where the transition zone between well mixed and stratified regimes was broad, so that movement of the front itself during the measurements presented fewer difficulties. Indeed, on one station North-west of Ushant the water column appeared with a 3 °C temperature inversion, without a corresponding stabilising salinity change, reflecting frontal movement. The temperature and chlorophyll *a* data shown in Figs 3 and 4 can be placed geographically by reference to Fig. 1. Here the maximum surface temperature gradient between stratified and well mixed regimes occurs in the neighbourhood of station B. Station G has been included as representative of the extensive area of stratified water that is established on the shelf in the Western Approaches during the summer months. High values of chlorophyll *a* occur in surface waters in the frontal region. These extend well into the stratified side, and are also observed in the thermocline. The visual correlation with the temperature section is conspicuous. It would be misleading, however, to suggest that the phytoplankton distribution is controlled by temperature. The common factor producing these distribution patterns is water turbulence.

Fig. 2 Continuous surface temperature, salinity and chlorophyll *a* traces obtained south of Ushant (see Fig. 1, legs 33, 34 and 35). Note that the chlorophyll *a* trace responds with the temperature gradient (or perhaps more correctly near where the second differential of temperature with respect to time (distance) takes a positive sign).



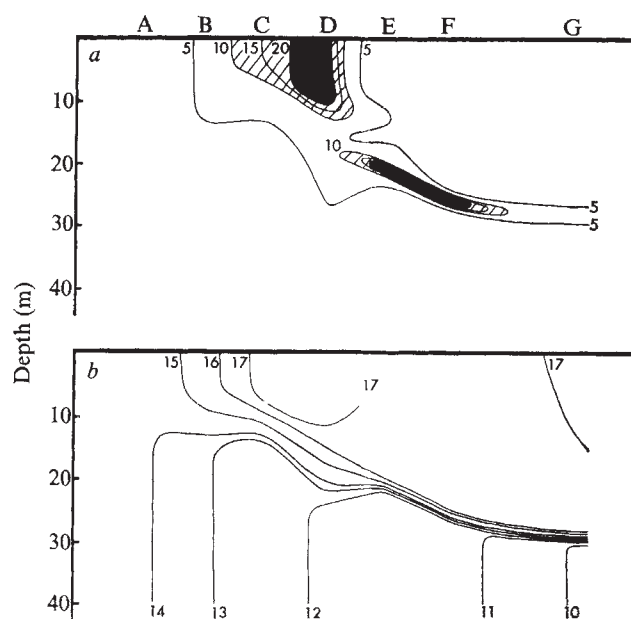


Fig. 3 *a*, Chlorophyll *a* section through the frontal region. The units of chlorophyll *a* concentration are mg m^{-3} . *b*, The corresponding temperature section. The units are in $^{\circ}\text{C}$. Station positions A, B, C, D, E, F, G are as shown in Fig. 1.

In the stratified region three different mixing regimes can be identified, namely, the top mixed layer, the thermocline and the bottom mixed layer. The turbulence in the top mixed layer is derived from wind stress and night-time convection. The turbulent kinetic energy production has its maximum input near the surface. In the bottom mixed layer the turbulent energy is derived mainly from the tides, but near the shore, swell motion may also make a significant contribution. In this region the turbulence declines away from the sea floor. The thermocline forms between the two mixed layers when the wind above the tide below can no longer stir the excess surface heating downwards. Here the ratio of the buoyancy flux to the turbulent energy production rate (that is, the local flux Richardson number) will be a maximum and the water column tends to stabilise, further inhibiting mixing. Towards the coast of France the tidal stream amplitude increases (Table 1) and the increased production rate of turbulent kinetic energy from below reduces the depth at which the thermocline can develop. The temperature section of Fig. 3 therefore represents the gradual change from the complete overlap which excludes the development of the thermocline to the situation where the wind and bottom mixed regimes are well separated vertically.

In general, differences in salinity will also contribute to the stability of the thermocline. The relative importance of salinity and temperature to the buoyancy flux can be determined from the ratio $\beta\Delta S/\alpha\Delta T$ where α is the coefficient of thermal expansion, β the coefficient of saline contraction and ΔT and ΔS are the corresponding temperature and salinity differences across the thermocline. This ratio was generally less than 5% (see Table 1) and so low that the temperature structure (Fig. 4) can also be considered as representative of the density structure.

In the stratified region, plant production in the surface layer is restricted by low levels of inorganic nutrients (Table 1, stations E, F, G). Values of chlorophyll *a* are also low below the thermocline, in spite of higher nutrient levels, because the mean light level is much reduced (generally less than 1% of the surface values). Why then are the values in the intermediate region in the thermocline as much as forty times larger than those above or below? Usual interpretations are based on phytoplankton aggregations due to sinking and settling on density discontinuities¹⁹, or active downward swimming of flagellates

towards the source of nutrients²⁰. By considering a time scale for mixing, it can be illustrated that the interaction of the turbulence level with the levels of light and nutrients may largely determine the distribution characteristics of the chlorophyll *a* profiles.

Dispersal of phytoplankton in well mixed and stratified regions

Estimates for the lifetime of small scale structures in the sea have led to some interesting speculations concerning their generation and dissipation^{21,22}. In a similar manner it is useful to have a diffusive time scale for the chlorophyll *a* structures in the water column. A characteristic time for a layer to mix above and below with its surroundings depends on the layer thickness, H , and the diffusion coefficient K . A time for mixing, t , may be defined by

$$t \sim H^2/4K$$

by analogy with the classical problem in heat flow, where t then represents the time taken for a uniformly heated layer to lose half its excess heat into a medium with the same conductivity²³.

In the bottom mixed layer the dominant stirring action of the tides produces a homogeneous temperature region in which the temperature gradient approaches the adiabatic value²⁴. If close to the bottom, the diffusion coefficient may be written as²⁵

$$K = 0.4u_*H$$

where the friction velocity u_* is defined from the bottom stress, τ_B , by $\tau_B = \rho u_*^2$, ρ is the density of seawater and H is the

Fig. 4 Vertical profiles of chlorophyll *a* and temperature obtained in: *a*, the well mixed (A); *b*, frontal (C); and *c*, stratified regions (E). The values are the original uncalibrated data which may be fixed absolutely by reference to Fig. 3.

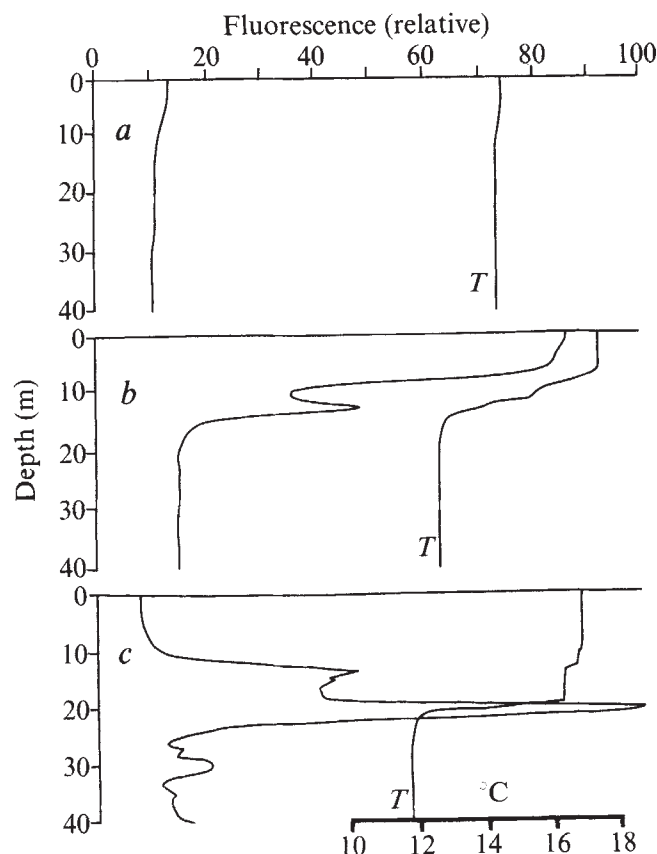


Table 1

Stations	A	B	C	D	E	F	G
Water depth (m)	92	98	107	108	106	94	110
Tidal stream amplitude at springs (m s^{-1})	1.3	1.1	0.9	0.8	0.7	0.7	0.6
Surface minus bottom temperature ($^{\circ}\text{C}$)	0.2	2.7	5.1	5.7	5.3	5.4	7.5
Surface minus bottom salinity (‰)	0.01	0.024	0.065	0.060	0.043	0.067	0.074
Buoyancy flux ratio ($\beta\Delta S/\alpha\Delta T$) (%)		4	5	4	3	5	3
$\text{PO}_4\text{—P}$ Surface layer 2 m	0.41	0.37	0.43	0.49	0.28	0.16	0.18
($\mu\text{g atom l}^{-1}$) Bottom layer 40 m		0.39	0.39	0.42	0.50	0.61	0.73
$\text{NO}_3\text{—N}$ Surface layer 2 m		2.56	1.79		0.1	0.69	0.25
($\mu\text{g atom l}^{-1}$) Bottom layer 40 m		0.58	1.39		3.2	0.85	1.39
$\text{SiO}_3\text{—Si}$ Surface layer 2 m	0.95	0.89	1.26		0.56	0.26	0.59
($\mu\text{g atom l}^{-1}$) Bottom layer 40 m		0.85	0.74		0.63	0.93	1.23

height above bottom, then a mixing time scale for the bottom region of height H is

$$t \sim H/u_*$$

Here the validity of this relationship away from the bottom has been extended for illustrative purposes for an order of magnitude estimate. The bottom stress was determined from current meters placed 1, 2 and 3 m off the bottom at position $49^{\circ}27'\text{N}$, $4^{\circ}42'\text{W}$ over a period of a month where the tidal streams were similar in amplitude to those in the neighbourhood of stations D and E. The semi-major axis of the semi-diurnal lunar, M_2 , friction velocity was $\sim 2 \text{ cm s}^{-1}$ (ref. 26). This gives a time scale for mixing in this region below the thermocline of the order of 1 h during maximum tidal streaming. This is small in comparison with the division rate of phytoplankton cells²⁷ and does not permit a chlorophyll-rich zone to become established below the thermocline where the light intensity is still favourable. Furthermore the efficiency of mixing in this region explains why the small scale structures in the chlorophyll a profiles in the region below the thermocline (Fig. 4c) are transitory and not generally reproducible from profile to profile. The mixing efficiency in the bottom layer makes hypotheses concerning the migration of plant cells into nutrient-rich water less attractive since these cells risk being rapidly dispersed in the same manner as heat. Similar calculations can be made for a time scale in the wind-mixed layer, but as conditions at the sea surface are unpredictable the derived mixing values are less meaningful.

A time scale for the thermocline can be formed from

$$t \sim \frac{H^2 \delta T / \delta z}{4F_h}$$

where F_h is the heat flux penetrating a thickness H of the thermocline of temperature gradient $\delta T / \delta z$

or
$$t \sim \frac{H(\theta_2 - \theta_1)}{4F_h}$$

where $\theta_2 - \theta_1$ is the temperature difference across the region H . Substituting typical values, $H = 5 \text{ m}$, $\theta_2 - \theta_1 = 4^{\circ}\text{C}$ and $F_h = 2 \text{ kcalorie cm}^{-2} \text{ month}^{-1}$ (based on the warming rate of the bottom mixed layers), gives a time scale t , of about a week. This is large compared with the cell division rate of most phytoplankton. Thus, higher cell concentrations in the thermocline may simply reflect the reduced tendency for their dispersal by turbulence. The large time scale in the thermocline is consistent with the persistence of the chlorophyll a maximum in the thermocline from station to station. Cells in this region would also be first in line for nutrients brought up from below in the same manner that heat is continually mixed downwards.

In the well mixed region (Fig. 4a) there are both adequate light and nutrients (Table 1) for cell growth at the surface. The thoroughness of the mixing (or the short time scale), however, does not give the plant cells sufficient time in the surface layers to produce any marked degree of surface phytoplankton

Fig. 5 Aerial photograph from 6,000 feet (August 7, 1975; 1400 GMT) showing red tide effects in the neighbourhood of station C. Surface wind light and variable; sea state—low swell.



concentrations here. In a similar manner, vertical structures in both chlorophyll *a* and temperature profiles are conspicuously absent in this region.

Conditions favouring summer plankton blooms

The frontal region represents an area of recently stabilised mixed water in which the combination of high nutrients and a shallow upper mixed layer create conditions suitable for the rapid growth of phytoplankton²⁸. These conditions are particularly dependent on weather and tide. At spring tides, for example, the tidal stream amplitude is approximately double that at neap tides, with a corresponding cubed production rate of turbulent kinetic energy in the bottom mixed layer, causing the well mixed region to increase in size at the expense of the more stratified region and nutrients to be brought to the surface. As the tidal streams slacken towards neaps, the surface water stabilises and conditions are set for a plankton bloom. In a similar manner, a period of turbulent weather will cause the frontal boundary to move into the stratified region, particularly if this is also the wind direction. Indeed a continual series of blooms may be created by the interaction of weather and tide as the balance between stabilisation and increasing turbulence is altered along this shallow thermocline region. It is worth remarking that it is the shallowness of the thermocline in this region that largely determines the size of the area likely to be effectively mixed and subsequently to produce favourable growth conditions. Our observations were made during calm weather following a period of spring tides and strong winds, which perhaps accounts for the large size of the region in bloom condition. The high surface values for dissolved inorganic nutrients at stations C and D, which exceeded those at 40 m (Table 1), cannot yet be explained.

Red tide characteristics within bloom region

Although so far we have not found it necessary to consider the swimming activities and buoyancy effects of the phytoplankton cells it is evident that they must be important on smaller scales within the bloom region. Dominant in the frontal region was the dinoflagellate *Gyrodinium aureolum* Hulburt, a species that has been reported as occurring in high concentrations in other areas²⁹⁻³¹. Counts of a representative sample (34 mg chlorophyll *a* per m³) gave the following values (cells per 1 × 10⁶): *G. aureolum*, 1.7; *Prorocentrum micans* Ehr., 0.01; flagellates, 1.4; and all other photosynthetic cells, 0.005. Areas were found where the concentration of chlorophyll *a* was as high as 100 mg m⁻³. A week later these reddish-brown streaks were observed from a height of 6,000 feet (Fig. 5), which were aligned in parallel but broken and irregular rows typical of a red-tide phenomenon³². A conservative estimate³³⁻³⁵ would suggest these patches contain more nutrients than were available in the water, and it seems unlikely that they could be produced without any interaction between cell movement and water turbulence. Concentrating mechanisms might be based on a daytime upward migration⁴³ coupled with weakly organised Langmuir circulations³⁶, or on the interaction between phytoplankton movement and internal waves³⁷. Red tide effects

have also been reported from inshore regions, though these are often due to positively buoyant organisms such as *Noctiluca*^{38,39}.

Primary production in characteristic regions

Interesting as these distributions of phytoplankton standing crop are, they are not a measure of primary production in the various regions. Rates of carbon dioxide fixation were determined by inoculating appropriate water samples with NaH¹⁴CO₃ and incubating them at sea surface temperature under fluorescent lights in the ship's laboratory or *in situ* under natural light at various depths. The data are summarised in Table 2. Mean values for chlorophyll *a* are representative for the vertically mixed regime in the neighbourhood of station A, for the frontal region between stations B and D, and for the top and bottom mixed layers in the stratified region. The thermocline is considered to be between 25 m and 30 m, and a mean value for chlorophyll *a* across the most concentrated 5 m of the chlorophyll peak is given. The assimilation index (AI)²⁷ is the potential for carbon fixation under light saturation (equivalent to 10% midday summer sunlight)⁴⁰. High indices were found for plant populations in the thermocline and in the vertically mixed regime. Lower indices for other regions may reflect a lack of dissolved nutrients or a poor physiological condition of the cells. In the frontal region the assimilation index seems to be inversely related to cell concentration, and for a higher chlorophyll *a* concentration of 78 mg m⁻³ a value of 1.34 mg carbon per h per mg chlorophyll *a* was observed.

Rates of primary production *P* were calculated from the equation

$$P = \text{AI chlorophyll } a \int_0^z I_0 e^{-kz} dz$$

(where *I*₀ is subsurface light intensity and *z* is depth of the water column) and expressed relative to a value of unity for the vertically mixed regime. In the stratified regime, for which the relative production rate is greater than one, the phytoplankton in the thermocline account for at least 50% of the total carbon fixation. Some caution is necessary in specifying this proportion since it will vary with light intensity. For example, under high surface light intensities photosynthesis will be maximal in the thermocline but saturated or light-inhibited in the top mixed layer. The production occurring below the thermocline seems relatively unimportant. In the frontal region, in spite of both the high extinction coefficient and the low assimilation index, the relative production is high. Thus, it seems that this weakly stratified region has an important bearing on primary production in the English Channel in the summer, and, furthermore, it is notable that the turbulent structure is also important in determining the rate of production of phytoplankton as well as the distribution pattern for the standing crop.

It is clear that further studies on primary production and the distribution of chlorophyll *a* in other associated frontal systems in the English Channel, for example, around the Lizard and Land's End (see Fig. 1), are necessary. Further work also needs to be directed towards answering questions such as which of

Table 2 Primary production data

	Mixed	Frontal	Wind mixed layer	Stratified Thermocline	Bottom mixed layer
Chlorophyll <i>a</i> (mg m ⁻³)	0.45	19.0	0.55	9.5	0.66
Extinction coefficient <i>k</i> (m ⁻¹)	0.12	0.34	0.10	0.1 < <i>k</i> < 0.3	0.10
Assimilation index (AI) (mg C fixed per h per mg chlorophyll <i>a</i>)	4.2	1.9	2.2	5.7	0.2
Relative production rate (<i>P</i>)	1.0	6.5	0.6	0.7-1.1 1.6	0.05

the three regions, well mixed, frontal or stratified, is generally the most productive; what are the relative contributions of the top mixed layer, thermocline and bottom mixed layer to the total production of the water column in the stratified regime; and how representative is a single station, like E1 (ref. 41), of production in the English Channel? The interrelationship between the distribution of phytoplankton and herbivorous zooplankton is poorly understood, particularly for the stratified regime. It seems probable that the high concentration of plant cells in the thermocline is an important food source for both migratory⁴² and non-migratory grazing organisms.

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Models for metal ion function in carbonic anhydrase

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A model catalyst is described which has properties in common with carbonic anhydrase. The model demonstrates the availability of a mechanism, previously only hypothetical, for the action of the enzyme. It also shows, however, that this mechanism alone is not adequate to produce the high activity of the enzyme.

DETECTED in 1928, discovered in 1932, recognised to contain zinc in 1939, obtained pure in 1959, crystallised in 1962 and the subject of some 1,800 papers and articles, carbonic anhydrase continues to elude a full mechanistic description, even though this enzyme is small, stable and easily isolated, and its X-ray structure is known to a resolution^{1,2} of 2 Å.

Carbonic anhydrase catalyses a range of reactions, including the hydration of carbon dioxide and aldehydes, and the hydrolysis of carboxylic, sulphonic and carbonic esters. The CO₂ hydration is apparently the reaction of biological importance, both in respiration and in intracellular CO₂–HCO₃[–] equilibration. In most of these reactions the rate in the absence of enzyme is believed to be limited by the nucleophilic attack of an oxygen atom or ion at an electrophilic centre, usually carbon, illustrated in Fig. 1a. The enzyme accelerates the CO₂ hydration, the acetaldehyde hydration and the hydrolysis of *p*-nitrophenyl acetate by factors of 10⁹, 10⁵ and 10⁵ respectively over the reaction in water.

The zinc ion which occurs naturally in carbonic anhydrase may be replaced artificially by other metal ions, but only cobalt(II) and zinc confer appreciable activity on the enzyme; with no metal or with other metals the enzyme is practically inactive in hydration.

The enzyme possesses an ionising group with a *pK_a* of

about 7; only the alkaline form of the enzyme is catalytically active.

One elegant mechanistic suggestion³ is that a metal-bound water molecule could ionise to provide the necessary nucleophile in the form of metal-bound hydroxide (Fig. 1b). Other possibilities include the ionisation of a nearby histidine group to provide a general-base catalyst at the active site⁴; the zinc could then act as a point of substrate binding, perhaps also polarising and thereby activating the substrate by virtue of its Lewis acid strength⁵. Whatever the mechanism of nucleophilic attack, other stages of the reaction may also be catalysed, such as proton transfer within the enzyme–substrate complex⁶ or between the enzyme and its aqueous environment⁷.

The purpose of this article is to re-examine the plausibility of the first mechanism referred to above, the zinc-bound hydroxide nucleophile (ZHN) hypothesis. Reviews and background literature are given in refs 2 and 8.

A priori arguments against the ZHN mechanism have been twofold: first, that a zinc-bound water cannot have such a low *pK_a* as 7 (refs 4 and 9); second, that a zinc-bound hydroxide ion would be so polarised by the zinc as to lose most of its negative charge to the zinc and thus be too weak a nucleophile to attack, for example CO₂ (refs 6 and 10 and Fig. 1b). Transition-metal hydroxy complexes can indeed attack CO₂ (refs 11 and 12), but so far few quantitative data are available.

To investigate the universality of these two presuppositions, model systems were chosen to reflect closely the desired properties of the enzyme. The much-abused term “model” is here understood as a simple chemical system with a property or properties relating it to the biological system under consideration. Ideally a range of model compounds, structurally controlled to reproduce in varying degrees the property in question, provide a quantitative scale on which the biological system

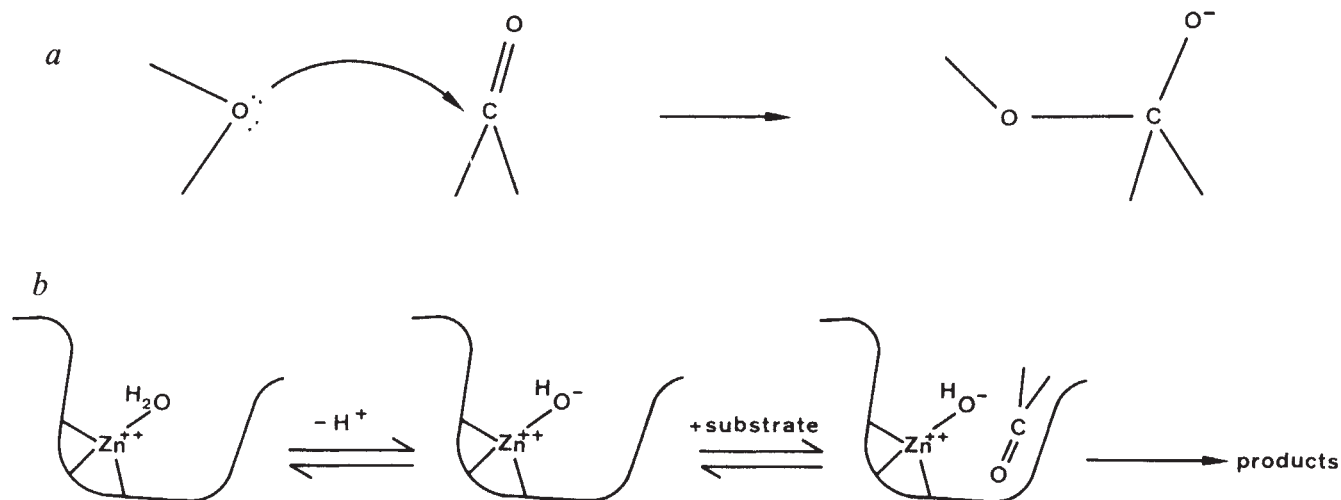


Fig. 1 Nucleophilic attack. *a*, Negative charge on oxygen enables it to react through its outer electron pairs with a positive centre such as the carbonyl carbon shown here; *b*, a postulated mechanism for nucleophilic attack in carbonic anhydrase.

might fall; if it continues to deviate from this scale then it has still not been fully understood. A model system may alternatively be designed to reproduce one of the biological properties of the system, for instance, to test the hypothesis that a sufficient condition for the action of an enzyme is a particular conjunction

of reacting atoms or groups; if successful this does not confirm that the biological system acts in the same way as the model, but it does show that the action of the simple system is in principle available for nature to make use of in the complex one.

An ionised water molecule?

Discussion of factors influencing the ionisability of water bound to zinc is bedevilled by the possible formation of hydrolysed cluster species and by the uncertain coordination number of the metal ion when water molecules are counted^{13,14}, as well as by the complexity of the equilibria present^{14,15}. To overcome these problems, macrocyclic ligands of the type CR (ref. 16 and refs therein) (Fig. 2) were used. Here the four nitrogen donor atoms are perforce bound to the metal, giving room for one or two water molecules. Electronic spectroscopy¹⁶ and X-ray crystallography¹⁷ support coordination numbers in aqueous solution of five for $CoCR^{2+}$ and $ZnCR^{2+}$, and six for $NiCR^{2+}$ and $CuCR^{2+}$.

Titration of the complexes in aqueous solution with sodium hydroxide reveals the pattern of behaviour shown in Table 1; two points emerge. First, the metals which can activate carbonic anhydrase can also promote the ionisation of water in their CR complexes. If this is due to the reduced coordination number of zinc or cobalt then the same ionisation should be possible in carbonic anhydrase. Second, nickel and copper do not promote this ionisation, although they are normally stronger Lewis acids, forming stronger complexes, than zinc or cobalt (II) (for example ref 18). A tempting conclusion is that whatever chemical influence determines the ionisation pattern of the CR complexes must also operate in carbonic anhydrase. This is, however, a *non sequitur*; a more logical approach is required.

The stronger acidity of five-coordinate $ZnCR^{2+}$ and $CoCR^{2+}$ than that of their corresponding hexaquo ions is probably determined by their reduced coordination number, as can be argued on simple electrostatic grounds^{13,19}, reducing the overall coordination number increases the total positive charge on zinc and the resulting polarisation of the bound H_2O . A further reduction in pK_a when the coordination number is reduced to four, as found in carbonic anhydrase¹, would therefore not be surprising. So far, carbonic anhydrase is the only zinc complex known for certain to have a coordination sphere composed of three nitrogens plus one oxygen in solution, although there are other candidates²⁰ for this. Similar titrimetric investigations using acyclic polyamine ligands point to the same correlation between coordination number and pK_a , (refs 21 and 22) with the additional insight that a less polar environment for the complex encourages ionisation still further,

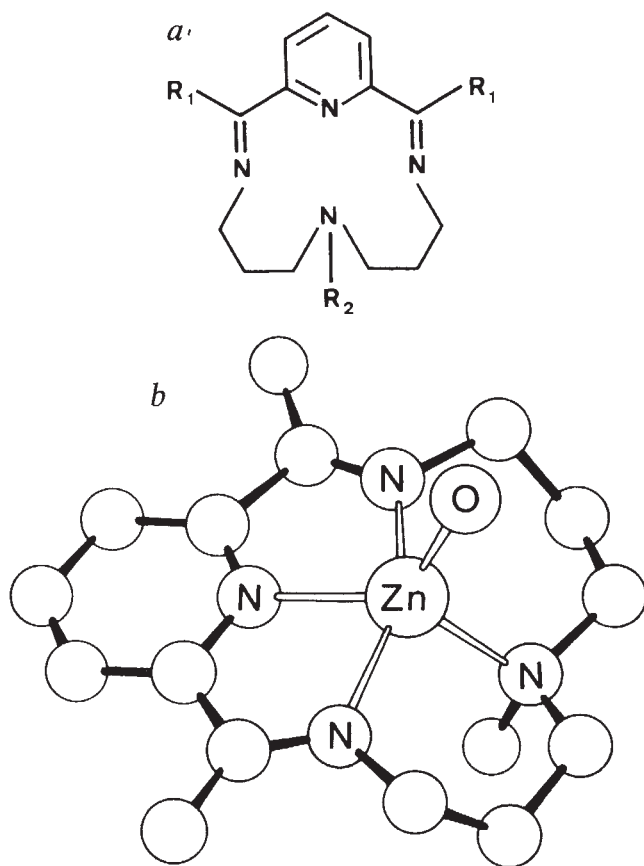


Fig. 2 Macrocyclic ligands. *a*, Free ligand. *b*, In the complex the four nitrogen atoms bind a metal ion— Zn^{2+} , Cu^{2+} , Ni^{2+} or Co^{2+} —which also binds water; the skeleton of the $Zn(N-MeCR)(H_2O)^{2+}$ complex is shown (H omitted, unlabelled atoms are C.) CR: $R_1 = CH_3$, $R_2 = H$; N-MeCR: $R_1 = R_2 = CH_3$; desdiMeCR: $R_1 = R_2 = H$.

perhaps to the extent of 1–2 units, as plainly shown by methylation of the NH_2 groups of the polyamine²³. This factor could well operate at the relatively non-polar active site of the enzyme. The provision of a firm binding site for zinc, of low coordination number and low polarity, and accessible to substrate, could indeed be the reason for the surprisingly large size of the protein in comparison with the substrate.

The probable higher coordination of the copper and nickel CR complexes similarly explains their lesser polarising effect on bound water molecules. Such a coordination number would reflect the known lesser propensity of zinc and cobalt, in comparison with copper and nickel, towards the formation of geometrically regular complexes, and indeed this very property, which imparts a greater coordination flexibility to Zn^{2+} and Co^{2+} , has been speculatively put forward as determinative of the activity of carbonic anhydrase with zinc and cobalt²⁴. The active site of carbonic anhydrase appears to be

poor a nucleophile to have a catalytic role in carbonic anhydrase. In addition, it was felt desirable to see what sort of modifications short of complexation by apocarbonic anhydrase would induce catalytic activity of any kind in zinc ions. The aim was thus to find a catalyst containing zinc ions along with organic groups and/or the hydroxide ion.

Carbonic anhydrase's range of substrates is wide, so it was necessary at the start to restrict the range of reactions examined. One reaction was studied intensively—the hydration of acetaldehyde. This was chosen as it is very efficiently catalysed by carbonic anhydrase and for reasons of experimental convenience.

The basic mechanism of acetaldehyde hydration has been well reviewed²⁵.

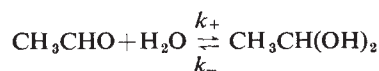


Table 1 Ionisation of water bound to metals in macrocyclic complexes, in aqueous solution at low ionic strength (0.002–0.04 mol l⁻¹)

Ligand	M	M(ligand)OH ₂ ²⁺ Temperature (°C)	K_a $\rightleftharpoons$	M(ligand)OH ⁺ + H ⁺ pK_a^*	Approximate heat of ionisation (kcalorie mol ⁻¹)
CR	Zn	25		8.69†	} 7.1 ± 1
CR	Zn	0		9.17	
CR	Cu	25		> 11	
CR	Ni	25		> 11	} 8.3 ± 1
CR	Co	25		8‡	
N-MeCR	Zn	25		8.12	
N-MeCR	Zn	0		8.68	} 6.0 ± 1
N-MeCR	Cu	25		> 11	
desdiMeCR	Zn	25		8.13	
desdiMeCR	Zn	0		8.53	} 6.0 ± 1
(H ₂ O) ₆	Zn, Co	—		~10	
Apocarbonic anhydrase	Zn, Co	—		~7	

* $pK_a = -\log_{10} K_a$. Error estimated from several determinations < 0.05 units.

† This supercedes the value given in ref. 19.

‡ Estimated value; apparent cluster formation prevented a more accurate determination.

fairly accommodating to the geometry preferred by the metal (ref. 25 and ref. 2, 642–643). Following this argument, one could say: if the activity in carbonic anhydrase is determined by an ionising metal-bound water molecule, then the non-activity of Cu^{2+} and Ni^{2+} in carbonic anhydrase could be a consequence of the non-ionisability or non-existence of this water, the latter a consequence of the coordination number taken up by Cu^{2+} or Ni^{2+} as the resultant of the different geometries preferred by the metal and the apoenzyme respectively. This must however be regarded as an hypothesis to be investigated and not as an explanation, particularly in view of the existence of an (as yet unidentified) ionising group in the inactive Mn^{2+} , (ref. 46) Cu^{2+} (E. Grell and A. McConnell, personal communication) and $(\text{VO})^{2+}$ (ref. 27) substituted enzymes.

The heat of ionisation of bovine carbonic anhydrase, 7 kcalorie mol⁻¹, has been taken as an indication that the ionising group is histidine, whose imidazole group has a similar heat of ionisation²⁸. The data in Table 1 shows that this value is equally compatible with the ionisation of zinc-bound water. The point of emphasis here, however, is the potential stability of a zinc-bound hydroxide ion, irrespective of whether it is formed by dissociation of a water molecule or, for example, by the addition of hydroxide accompanying a conformational change of the protein.

A zinc-bound hydroxide nucleophile?

The purpose of the kinetic studies here was to test the second presupposition above—that metal-bound hydroxide is too

In contrast to carbonic anhydrase, aqueous zinc ions are only poor catalysts for the reaction, but there is a very simple, general catalytic effect in which zinc ions and other species in solution work together to catalyse the hydration reaction³⁰. These other species include in particular hydroxide ions, and this effect was investigated further in the hope that it might also throw some light on the ZHN hypothesis. Unfortunately, the cooperation between zinc and hydroxide ions is less amenable to systematic mechanistic study, as practical difficulties are caused by polynucleation and precipitation. To study this reaction further it was therefore necessary to use a different system containing zinc and hydroxide, and the natural choice was the ZnCR^{2+} complex, which was already available and whose properties were well investigated.

The efficiency of a catalyst in acetaldehyde hydration is defined as $dk_+/d[\text{catalyst}]$. Experimental details of the procedure used to measure the rates are given elsewhere (ref. 30 and refs therein). At low pH the efficiency of ZnCR^{2+} is low, but at high pH it becomes very high (Fig. 3). This behaviour superficially resembles that of carbonic anhydrase³¹.

Mechanism of ZnCR^{2+} catalysis

The efficiencies of various catalysts are shown in Table 2. Empirically, ZnCR^{2+} is a poor catalyst (Fig. 3, low pH) and ZnCR.OH^+ , the form of the complex predominating at high pH, a good one (Fig. 3, high pH). Thus the ZnCR^{2+} and OH^- cooperate to produce an enhanced catalysis, just as hoped for. A detailed analysis of the kinetics and activation parameters

(to be published) shows that the hydration proceeds through collision between ZnCR.OH^+ and CH_3CHO . Two possible mechanisms for this are shown in Fig. 4. Borate is a more powerful base than ZnCR.OH^+ , yet its catalytic effect is much smaller (Table 2). Therefore ZnCR.OH^+ cannot simply be acting as a general base (Fig. 4a), as then borate would be the better catalyst; it must be attacking the carbonyl group directly (Fig. 4b). Space-filling models suggest that simultaneous binding to zinc of hydroxide and aldehyde is forbidden by the geometry of the CR ligand; this point is being further investigated.

Table 2 Catalytic efficiency in acetaldehyde hydration

Catalyst	Efficiency	Reference
CH_3COO^-	0.023	30
CH_3COOH	0.073	
Zn^{2+}	0.0084	
$\text{H}_2\text{O}^{\text{a}}$	0.00014	
ZnCR^{2+}	0.036	
CuCR^{2+} (pH 5.0, 7.5)	0	This work
CuCR^{2+} (pH 9.5)	2 ± 1 (?)	
ZnCR.OH^+	196	
Zn(N-MeCR).OH^+	112	
$\text{Zn(desdiMeCR).OH}^+$	130	
OH^-	58,000	32
Carbonic anhydrase	14*	31
Borate	$\lesssim 7$	This work

Units are $\text{mol}^{-1} \text{l s}^{-1}$.

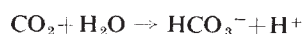
*The turnover number (700 s^{-1}) of the enzyme is divided by 50 mol l^{-1} , to obtain an order-of-magnitude correction for the presumed high concentration of catalyst at the active site.

To obtain a comparison, the catalytic properties CuCR^{2+} , NiCR^{2+} and CoCR^{2+} were examined in the same way. Unfortunately the nickel and cobalt complexes reacted directly with the acetaldehyde (shown by ultraviolet spectroscopy) and so no conclusion could be reached in these cases. CuCR^{2+} was unaffected by the acetaldehyde and showed no catalytic properties at pH values of 5.0, 7.5, or 9.5. This is consistent with the absence of a CuCR.OH^+ species in this pH range, demonstrated by titration (Table 1).

That the catalysis in the presence of CR involves direct attack of zinc-bound hydroxide backs up the hypothesis that the same attack occurs in the above-mentioned cooperative catalysis by zinc and hydroxide ions.

Carbon dioxide hydration

The availability of zinc-bound hydroxide as a good nucleophile provides an obvious next step for investigation of its catalytic ability in other reactions, particularly those catalysed by carbonic anhydrase. Most interesting would be the hydration of CO_2 , for which carbonic anhydrase's catalytic power is the greatest. This was examined qualitatively by mixing saturated CO_2 solutions with buffered solutions of ZnCR^{2+} at 0°C . The initial rate of pH change was measured and converted by comparison with an HCl titration to the rate of liberation of protons; this gave the reaction rate, for the stoichiometry is



at the pH used (9.2). The empirical catalytic constant of the ZnCR.OH^+ complex is given by

$$\frac{1}{[\text{CO}_2]} \cdot \frac{d[\text{CO}_2]}{dt} \cdot \frac{1}{[\text{ZnCR.OH}^+]} \quad (3)$$

and was determined as about $25 \text{ mol}^{-1} \text{l s}^{-1}$. Since previous work has demonstrated the absence of general-base catalysis in the hydration of CO_2 (refs 33 and 34) the most obvious

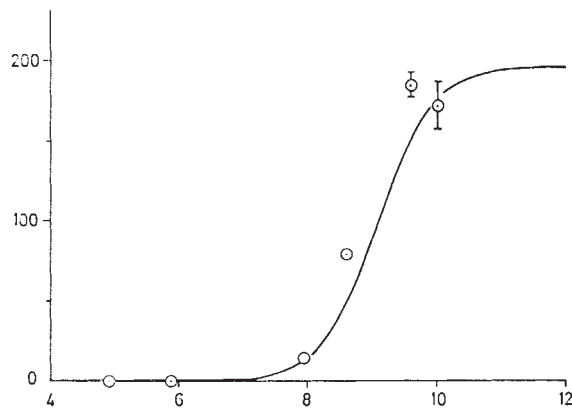


Fig. 3 The catalytic efficiency of ZnCR^{2+} in the hydration of acetaldehyde, as a function of pH. The solid line is the curve calculated assuming that the active species is ZnCR.OH^+ with an efficiency of $196 \text{ mol}^{-1} \text{l s}^{-1}$. The pK_a found is 9.07 (compare the value from titration, 9.17, Table 1). Increasing errors at high pH made measurement above pH 10 impossible.

mechanism available for attack is analogous to that of Fig. 4b. ZnCR.OH^+ is quite a good catalyst (Table 4), although still not comparable with carbonic anhydrase.

A similar result has been reported¹² using the complex $\{\text{Cu.glycylglycinate.OH}\}$, in which the active species was likewise considered to be a metal-bound hydroxide ion. ZnCR.OH^+ is a less ambiguous catalyst to interpret, as no potentially active ligand groups other than OH^- are present, and the metal has no further free coordination sites.

Relationship between model and enzyme

The applicability of the enzyme model to the enzyme can now be considered in relation to the ZHN hypothesis.

The titrimetric studies on ZnCR^{2+} and related complexes, and similar studies on a range of polyamines (refs 22, 23 and unpublished), make it clear that the ionisation of zinc-bound water is to be expected, on condition that the coordination number of zinc be reduced from six to five or four—a condition fulfilled by carbonic anhydrase. This does not prove that the ZHN in carbonic anhydrase exists, but it means, should a zinc-bound water in carbonic anhydrase be shown not to ionise, that this in itself would be a remarkable property of the enzyme, and in need of special explanation.

Table 3 Catalytic efficiency in carbon dioxide hydration

Catalyst	Efficiency	Reference
H_2AsO_3^-	0.9	33*
HAsO_4^-	0.002	
BrO^-	2	
SeO_3^-	1.2	
SO_3^-	0.2	
NO_2^- , NO_3^- , CO_3^{2-} , SO_4^{2-}	0	34*
Complexes MX_n , where $\text{M} = \text{Mn, Fe, Co, Ni, Cu, Zn}^{2+}$ and $\text{X} = \text{EDTA, nitrilotriacetic acid, dipyriddy, cysteine, mercaptoethylamine and cyanide}$	0	
$\text{Cu(glycylglycinate).OH}$	20	
ZnCR.OH^+	25	
Carbonic anhydrase	$10,000^\dagger$	
		35

Units are $\text{mol}^{-1} \text{l s}^{-1}$.

*In these references different units are used. The data have been converted so as to accord with the definition given in this article.

[†]See footnote to Table 2.

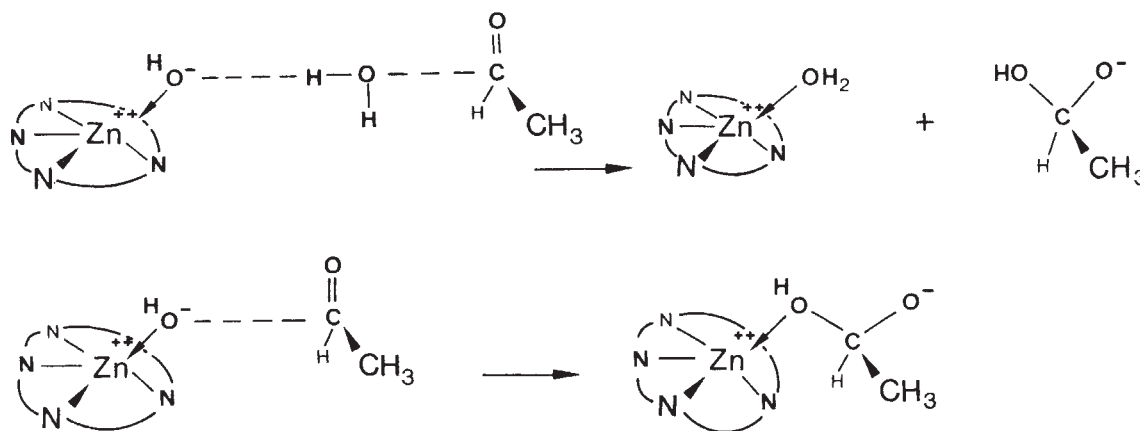


Fig. 4 *a*, General-base and *b*, direct (nucleophilic) attack by ZnCR.OH^+ . Only the rate-determining step is shown; proton transfer and ligand replacement at zinc are assumed to be fast.

The kinetic work has shown that a zinc-bound hydroxide ion can act as a very powerful nucleophile, even though its proton affinity is about 10^7 times that of a free hydroxide ion. Its nucleophilicity is great enough to account for the enzyme's catalysis of acetaldehyde hydration, though not of carbon dioxide hydration (Tables 2 and 3). The existence of this nucleophile in the enzyme and in the model does not mean that the mechanisms in the enzyme and in the model are the same; indeed, the rates for CO_2 are so different that, even if there is a ZHN in carbonic anhydrase, the enzyme must still have an extra means of assisting the hydration which the acetaldehyde, because of its different structure, cannot utilise. There is also evidence^{36,37} that an additional, tightly-bound bicarbonate ion could have such a role by mediating proton transfer in the zinc-substrate complex.

The evidence for the ZHN postulate on the basis of enzyme studies is far from overwhelming; model studies cannot add to it directly, although they appear to increase the previous plausibility and the comprehensibility of such a mechanism. What happens when one tries to extend the hypothesis to the metal-substituted carbonic anhydrases, with the thought that their activity or inactivity could be related to the presence or absence of an ionising water molecule at the metal? At first sight this seems reasonable. The flexible ions Zn^{2+} and Co^{2+} accept the distorted geometry and low coordination number offered by the enzyme, while copper and nickel do not, thus failing to polarise the bound water sufficiently to induce ionisation. Manganese is coordinatively flexible, but polarises bound water only weakly (it forms in general comparatively weak complexes¹⁸, and in carbonic anhydrase seems to be less capable than zinc of polarising sulphonamide inhibitors³⁸).

Before such a theory could be accepted, however, several obstacles would need to be removed. (1) Ionising groups are associated with metal in Mn^{2+} , Cu^{2+} and $(\text{VO})^{2+}$ -substituted carbonic anhydrases (see above). These would have to be shown not to be metal-bound water. (2) In the acid form of cobalt-carbonic anhydrase the metal ion is apparently very poorly accessible to exchanging water molecules³⁹. Also, the spectroscopic change associated with the activity-linked ionisation in the cobalt enzyme is very marked⁹, whereas the spectroscopic effects of water and hydroxide ligands are almost identical⁴⁰, and the spectrum of CoCR.OH_2^{2+} is correspondingly similar to that of CoCR.OH^+ (unpublished). Thus, even if the ZHN hypothesis is correct, certain features of the metal's coordination geometry require clarification. (3) A kinetic effect remains unexplained. The forms of zinc-carbonic anhydrase with the more acidic proposed zinc-bound water molecules—having presumably the most strongly polarising zinc ions—should by virtue of the diminished negative charge on the hydroxide's oxygen atom possess the weakest nucleophiles, with the smallest rate of attack on substrates. The reverse order is seen, how-

ever^{41,42}. This is also the case with the simple compounds $\text{ZnOH}^+_{\text{aq}}$ and ZnCR.OH^+ (Table 2) for the substrate acetaldehyde. Nucleophilicity and basicity are not always closely related⁴², but a satisfactory explanation of this would still be welcome.

Conclusions from models

Models show that the presence of the postulated zinc-bound hydroxide ion in carbonic anhydrase at neutral pH values would be in accord with reasonable expectation. This hydroxide ion would have sufficient nucleophilic power to account for the enzyme's activity in acetaldehyde hydration, although not in carbon dioxide hydration. The ZHN postulate for the enzyme led to the observation of surprisingly high nucleophilicity in the zinc-bound hydroxide. Thus the uses and limitations of chemically modelling a biological system are illustrated.

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Conservation of cytoplasmic poly(A)-containing RNA in mouse and rat

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By comparing the melting temperature of DNA-DNA duplexes between related species, it is shown that total single-copy DNA evolves at a faster rate than DNA which is transcribed into poly(A)-containing RNA. Such a comparison suggests that at least 70% of rat single-copy DNA does not code for protein.

THE organisation and function of the DNA in the eukaryotic genome are the subject of much speculation and research^{1,2}. There exists a variety of models which concern themselves with the regulation of gene activity. At present, however, few data show what fraction of eukaryotic DNA is genetically active, that is, what fraction of the DNA codes for protein. An alternative way of posing the same question is: how many different proteins are synthesised throughout the life of the organism?

Various estimates of this number make distinctly different predictions. Based largely on genetic analyses of *Drosophila* lethal mutations, the minimal estimate gives

approximately 5,000 genes encoded by the *Drosophila* genome³. Mammalian haploid genomes contain 3 pg of DNA⁴. Approximately 70% of the genome is single-copy DNA, known to be the site of transcription of most of the messenger RNA. Assuming a number-average molecular weight of 6×10^5 for eukaryotic mRNA⁵, 5,000 different mRNAs (the minimal estimate for *Drosophila*) represent only 0.5% of the single-copy fraction of mammalian DNA. If the number of genes is proportional to the genome size, this estimate for mammals increases to 50,000 different mRNA species and 5.0% of the available single-copy DNA. From a consideration of the mutation rate observed for amino acid changes in protein, Kimura has concluded that the cost to a species due to deleterious mutations would be very high if all the DNA of eukaryotes were expressed genetically⁶, in accordance with these low estimates of the protein coding fraction of the genome. Alternatively, there may be many more than 50,000 different mRNA species and, therefore, much more than 5% of the single-copy DNA of mammals may be protein-coding DNA. In the extreme,

Table 1 Extents of reaction and thermal stabilities of homologous and heterologous hybrids with rat ³H-cDNA and rat ³²P-scDNA

Experiment	Homologous						Heterologous					
	Reassociation temperature (°C)	Rat DNA % Rxn.	scDNA % Rxn.	ΔT _m (°C)	cDNA % Rxn.	ΔT _m (°C)	Mouse DNA % Rxn.	scDNA % Rxn.	ΔT _m (°C)	cDNA % Rxn.	ΔT _m (°C)	
a	60	90	86	0.5	87	3.0	90	67	14.0	82	8.0	
b	70	85	83	0.5	72.5	3.0	78	31	9.0	65	6.0	
c	73	75	73	0.5	67	2.5	74	18.5	8.0	51	6.0	
d	75	78	77.5	0.5	70	3.0	72	9.5	6.0	35.5	6.0	
e	75	—	—	—	—	—	—	1.3	—	4.0	—	
f	75	—	—	—	—	—	—	1.6	—	—	—	
SI Nuclease												
	Homologous						Heterologous					
	Reassociation temperature (°C)	scDNA % Rxn.	T _m (°C)	cDNA % Rxn.	T _m (°C)		scDNA % Rxn.	T _m (°C)	cDNA % Rxn.	T _m (°C)		
g	75	56	66.5	65	66.5		7	64.5	37	64.5		

The data for experiments a, b, d, and g are taken from Figs 1, 2, 3, and 4, respectively. Experiment c is identical to experiments a, b, and d with reassociation temperature of 73 °C. In experiments e and f, radioactive tracer DNA was incubated in the absence of non-radioactive driver DNA as in Fig. 1.

10^6 different mRNA species represent 100% of the single-copy fraction.

A direct test of these two hypotheses is technically difficult. It would be necessary to isolate and quantify all the different protein molecules (or mRNA molecules) synthesised in every tissue, at every stage of development throughout the lifetime of the organism. In experiments of this nature, it would be extremely easy to miss a relatively rare set of gene products. This class might well represent much, or even most, of the potential genetic complexity in the genome. Any number generated by direct counting of gene products in this manner is, therefore, perforce a lower limit.

We have now used an indirect approach to distinguish between these two extreme models. By comparing the melting temperature of DNA-DNA duplexes between related species, Kohne⁶ and Laird *et al.*⁷ have shown that the rate of base substitutions is high in mammalian evolution. Similar studies of mRNA-cDNA duplexes (complementary DNA synthesised with reverse transcriptase) have shown that haemoglobin mRNA evolves at a rate considerably slower than single-copy DNA⁸. This observation, if true for most or all mRNA sequences, would suggest that the reduced base substitution between two related species (in this case *Mus musculus* and *Rattus norvegicus*), when compared with the base substitution in total single-copy DNA, could be used to estimate the percentage of single-copy DNA homologous to mRNA in these organisms. We have confirmed and extended this observation by examining the thermal stability of heterologous cDNA-DNA duplexes in which the cDNA was synthesised from total cytoplasmic poly(A)-containing RNA isolated from a tissue culture cell line, rat myoblasts. This cDNA, representing thousands of different messenger RNA sequences, was annealed in vast DNA excess to mouse DNA^{9,10}. The comparison of the extent of reaction and thermal stability of these heterologous rat cDNA-mouse DNA duplex molecules with rat single-copy DNA-mouse DNA duplex molecules suggests that most of the single-copy DNA in these organisms does not serve as template for messenger RNA.

Single-copy DNA and cDNA homology

The relationship between thermal stability and degree of base mismatch of double-stranded DNA has been extensively investigated¹¹. The mean temperature of elution of duplex DNA from hydroxylapatite (T_m), compared with an internal standard of perfectly matched duplex DNA, is an indication of the degree of base mismatch. A reduction in T_m (ΔT_m) is proportional to the number of mispaired bases present. When molecules from two related species are compared by hybridisation, this ΔT_m represents the proportion of nucleotides which have been substituted in the DNAs since their divergence from the most recent common ancestor. Rat ³H-cDNA and rat ³²P-single-copy DNA (scDNA) were annealed with a vast excess of homologous (rat) or heterologous (mouse) DNA to a C_{ot} of 10^4 mol l⁻¹ in 0.24 M phosphate buffer (PB). At a temperature of incubation of 60 °C, 85–90% of the homologous reannealed DNA is retained by hydroxylapatite at 60 °C and elutes at a characteristic and reproducible temperature (Fig. 1a and c). The rat ³²P-scDNA, reannealed to cold rat DNA, elutes at the same temperature (± 0.5 °C) as the reannealed non-radioactive rat DNA "driver". The ³H-cDNA-DNA duplexes elute approximately 3 °C lower than the ³²P-scDNA-DNA duplexes. The reason for this reduced melting profile of cDNA-DNA duplexes is not understood. It is possible that the cDNA has a low enough molecular weight to account for this result as its average size as determined by alkaline sucrose gradient sedimentation (370 nucleotides) is smaller than that of the scDNA and the non-radioactive

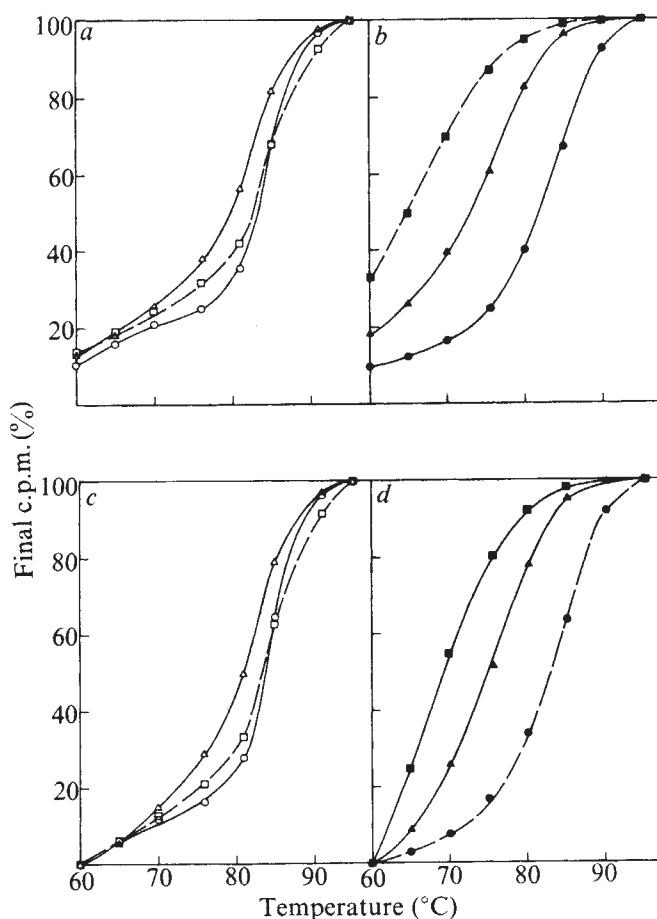


Fig. 1 Melting curves of hybrids of rat ³H-cDNA and ³²P-scDNA to mouse and rat DNA incubated at 60 °C and assayed on hydroxylapatite. Rat ³H-cDNA and rat ³²P-scDNA were hybridised to a vast excess (a ratio of at least 400) of mouse and rat DNA in 0.24 M phosphate buffer (PB) at 60 °C. The DNA, at 10 mg ml⁻¹, was denatured and incubated for 80 h, to a C_{ot} of $\sim 10,000$ in 0.24 M PB at 60 °C. 200 μ g of DNA, containing approximately 5,000 c.p.m. of both ³H-cDNA and ³²P-scDNA was diluted to 0.12 M PB and applied at 60 °C to columns containing 500 mg hydroxylapatite and 500 mg cellulose. DNA was eluted with 3 aliquots of 10-ml 0.12 M PB at 60 °C. The temperature of the columns was raised in 5 °C intervals and the DNA eluted with 10 ml of 0.12 M PB at each temperature. A final 10-ml elution was performed with 0.4 M PB at 95 °C. Recovery from the column was $100 \pm 5\%$. The amount of driver mouse and rat DNA was determined by the absorbance at 260 nm of each sample. ³H-cDNA and ³²P-scDNA in each sample was measured by scintillation counting of samples after precipitation with TCA and BSA carrier. DNA, and cytoplasmic poly(A)-containing RNA, were prepared as described previously¹⁶. ³H-cDNA was prepared from poly(A)-containing RNA with the reverse transcriptase of avian myeloblastosis virus. ³²P-scDNA was prepared by reassociation of total DNA to C_{ot} 200 in 0.24 M PB at 70 °C and removal of repeated sequences on hydroxylapatite. This 60% of the DNA, recovered in the single stranded fraction, displayed single-copy kinetics when rehybridised with total DNA. ³H-cDNA, complementary to single-copy sequences, was prepared by removal of repeated sequences by hybridisation to C_{ot} 20-DNA-Sepharex as previously described¹⁶. The unhybridised cDNA (90% of the total) when reannealed to total DNA showed no repetitive sequences¹⁶. The removal of the repetitive component of cDNA caused a marked depletion of the more abundant low complexity sequences. The remaining abundant sequences represented at most 20% of the single-copy cDNA¹⁷. Results are presented in integral form, that is as the percentage of total c.p.m. or absorbance having eluted up to each temperature. *a*, Thermal elution of rat DNA driver (○); rat ³²P-scDNA (□) and rat ³H-cDNA (△); *b*, Thermal elution of mouse DNA driver (●); rat ³²P-scDNA (■); and rat ³H-cDNA (▲); *c*, As panel *a* with the data normalised to 0% at 60 °C; *d*, As panel *b* with the data normalised to 0% at 60 °C.

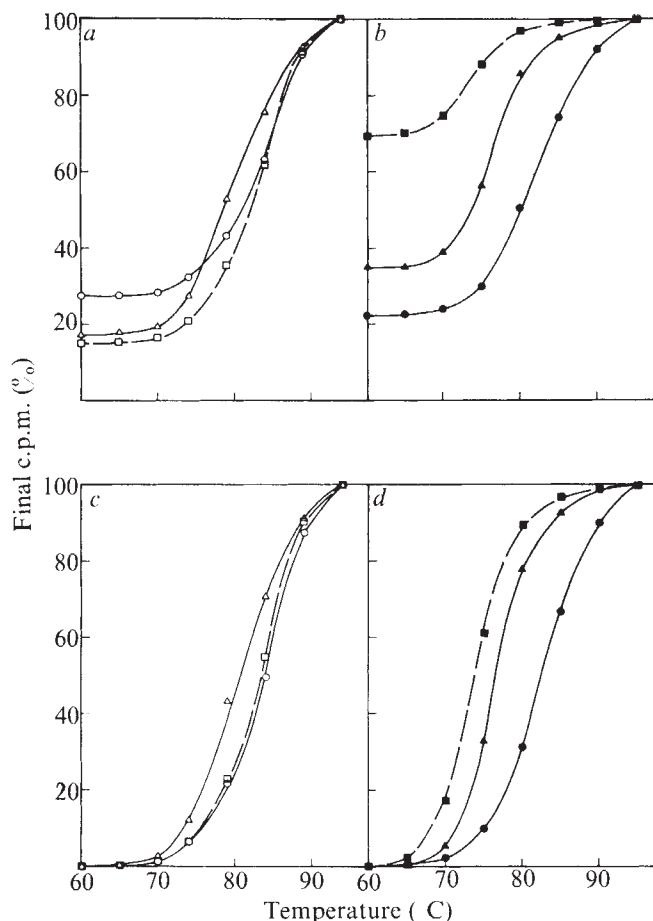


Fig. 2 Melting curves of hybrids of rat ^3H -cDNA and rat ^{32}P -scDNA to mouse and rat DNA incubated at 70°C and assayed on hydroxylapatite. Conditions were as in Fig. 1 except that the incubation was at 70°C .

DNA driver (450 nucleotides). Alternative interpretations include a base composition difference between cDNA and total DNA and low repetition frequency of a fraction of the "single-copy" cDNA.

In contrast, both radioactive probes, when annealed to heterologous (mouse) DNA in identical conditions, have the ability to bind to hydroxylapatite in standard conditions (Fig. 1b), 82% for ^3H -cDNA and 67% for ^{32}P -scDNA as compared with 87% for both probes in the homologous reaction.

In addition, both rat cDNA and rat scDNA, when annealed to mouse DNA, elute at considerably reduced temperatures relative to homologous duplexes (Fig. 1c and d, and Table 1). Moreover, the ΔT_m of the rat scDNA-mouse DNA duplex (14°C) is considerably greater than the ΔT_m of the rat cDNA-mouse DNA duplex (8°C). This difference suggests that these two fractions of the genome are not identical; there exists considerably more mismatching and therefore evolutionary divergence between rats and mice in the total single-copy fraction of the genome than in the fraction of the genome complementary to the cDNA probe.

A ΔT_m of 14°C represents a base substitution frequency of approximately 9% (ref. 11). Considering the moderate degree of stringency in this experiment, these data agree quite well with previously published reports which examined the relationship between rat and mouse single-copy DNA^{6,7}. In contrast, it is relatively difficult to equate the 8°C ΔT_m of rat cDNA-mouse DNA duplexes to a degree of base substitution because of the 3°C ΔT_m of rat cDNA duplexes. Without accounting for this homologous ΔT_m , the data

suggest a 5% degree of mismatch between the rat and mouse sequences represented in cDNA. Subtracting this homologous ΔT_m yields a value of 4%. Consequently, we conclude that the rat cDNA-mouse DNA duplexes are between 4 and 5% mismatched.

Estimations of coding DNA

There are two methods of determining the proportion of rat scDNA which has the same T_m as the cDNA sequences. From the data in Fig. 1, it can be deduced that roughly 30% of the scDNA melting curve overlaps the cDNA melting curve and, therefore, has the same extent of divergence as mRNA.

The other method consists of establishing annealing conditions which allow only duplexes of little divergence to react. Incubation at elevated temperatures discriminates against molecules which are poorly matched and selects for molecules which have undergone little evolutionary change. When the temperature of incubation is increased to 70°C , there is little effect on the extent of homologous duplex formation or on the T_m of the hybrids formed (Fig. 2a and c). In contrast, the formation of heterologous duplex molecules with rat scDNA is markedly reduced. The fraction of rat scDNA which is retained by hydroxylapatite after incubation at 70°C (Fig. 2b) is decreased to 30%; there is, however, only a small reduction in rat cDNA-mouse DNA duplex formation on increasing the temperature of incubation from 60 – 70°C . These data are consistent with the original observation (Fig. 1) that there exists considerably more mismatching in rat scDNA-mouse DNA duplexes than in rat cDNA-mouse DNA duplexes.

Also consistent is the fact that the T_m of the rat scDNA-mouse DNA duplex molecules formed at 70°C is considerably higher than the T_m of the duplex molecules formed at 60°C , that is, the reduced fraction of duplexes which do form at 70°C exhibit less mismatching than those formed in a less stringent criterion.

When the temperature of incubation is raised to 75°C , the same trend continues. The homologous annealing between rat scDNA or rat cDNA and total rat DNA driver is only marginally affected by this increase in the temperature of incubation. The heterologous experiments are markedly affected. The extent of reaction is decreased, and the T_m of the duplexes is increased. At a temperature of incubation of 75°C , only 9% of the rat scDNA forms duplex with mouse DNA. In identical conditions, 36% of the rat cDNA forms duplexes with mouse DNA (Fig. 3b). This represents a greater than sevenfold decrease in duplex formation for rat scDNA as compared with that observed at 60°C but represents less than a 2.5-fold decrease for the rat cDNA. Moreover, at this stringent criterion, the T_m of the rat cDNA-mouse DNA duplex structures is identical to the T_m of the rat cDNA-mouse DNA duplexes (Fig. 3d).

It is possible that only a fraction of the nucleotides that bind to hydroxylapatite after incubation at 75°C are in duplex form. This possibility predicts that only a fraction of each cDNA molecule and, therefore, only a fraction of each rat mRNA molecule from which it is synthesised, is relatively homologous to mouse DNA. To examine this possibility, the structures formed after incubation at 75°C were assayed with S1 single-stranded nuclease¹² (Fig. 4). In all cases the extent of duplex formation is similar to the identical experiment as assayed with hydroxylapatite (Fig. 3). In particular, after incubation with mouse DNA, only 7% of the rat scDNA is nuclease resistant. But, 35% of the rat cDNA is nuclease resistant. In addition, the T_m s of these duplex structures are identical, as are the T_m s when assayed with hydroxylapatite (Fig. 3d). These data confirm our interpretation that the majority of single-copy DNA diverges more rapidly than mRNA sequences.

The distribution of divergence in the two populations of

molecules is very different. Total single-copy DNA has a broader spectrum of sequence divergence than protein-coding sequences. The upper limit of its distribution overlaps the tighter distribution of the cDNA sequences though the distribution is weighted toward diverged sequences. Conditions which select for sequences with the same distribution will reduce the formation of heterologous rat scDNA duplexes more than the heterologous cDNA duplexes. If the extent of scDNA duplex formation at 75 °C is normalised to the cDNA duplex formation at 75 °C, the proportion of the scDNA sequences with an identical T_m is 18%. We feel that these data indicate that at most 20–30% of the scDNA of the rat has the same degree of divergence observed for mRNA sequences. There should be some single-copy DNA with greater than average sequence conservation due to stochastic processes in mutation; in addition, there might be functions other than protein synthesis for which conservation of sequences is important. Consequently, this number is very much an upper limit for the potential protein-coding DNA.

It is crucial to ascertain to what extent the properties of this cDNA probe are representative of the sequence properties of general messenger RNA. When hybridised in RNA excess to its template poly(A)-containing RNA³, the cDNA is rendered double stranded with kinetics which suggest that there are about 6,000–7,000 different mRNA sequences¹⁴. These data also show that there is no very low complexity component in the mRNA which may render the cDNA probe unrepresentative; and that the more abundant sequences in the mRNA represent at most 20% of the cDNA probe and cannot thus be responsible for the extensive cross hybridisation observed. RNA excess

Fig. 3 Melting curves of reassociations of rat ³H-cDNA and rat ³²P-scDNA to mouse and rat DNA incubated at 75 °C and assayed on hydroxylapatite. Conditions were as in Fig. 1 except that the incubation was at 75 °C.

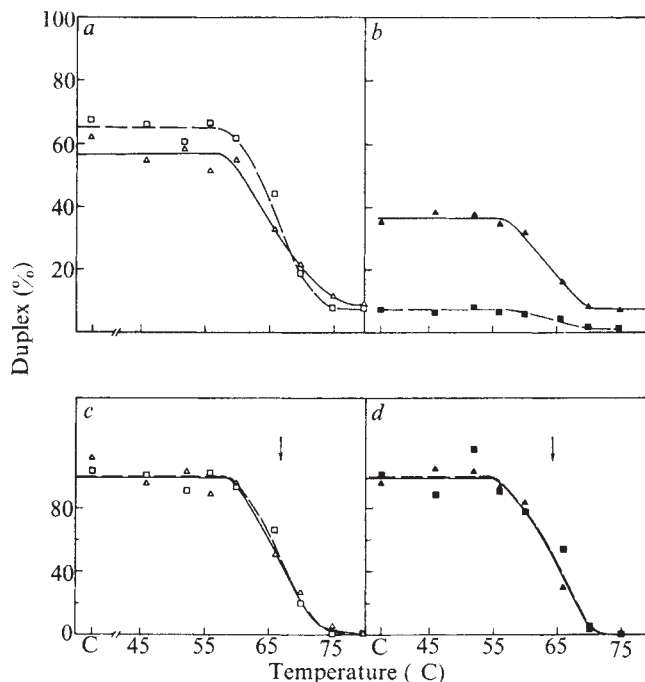
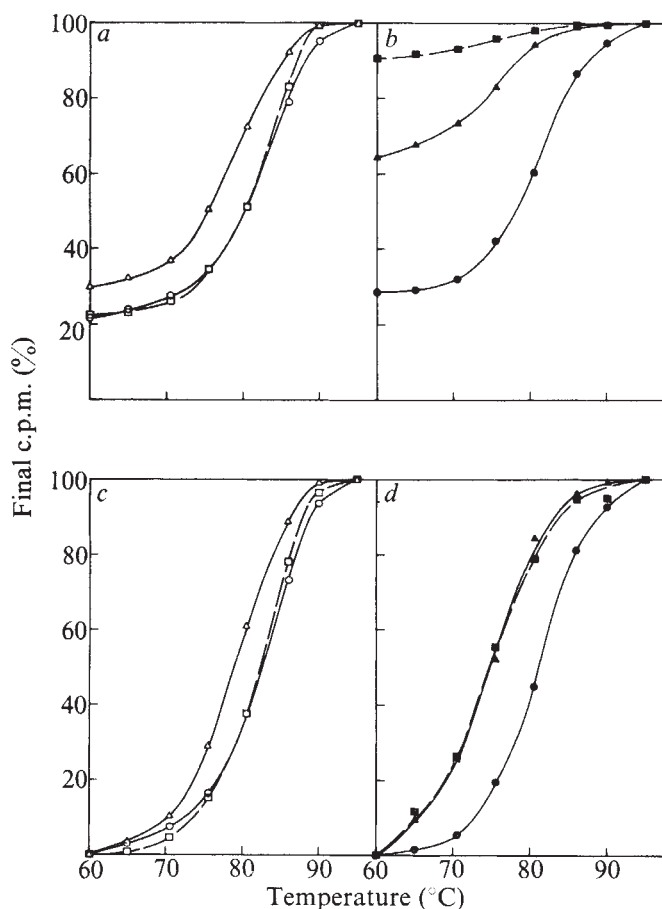


Fig. 4 Melting curves of reassociations of rat ³H-cDNA and ³²P-scDNA to mouse and rat DNA incubated at 75 °C and assayed with S1 nuclease. Reassociations were carried out in 0.24 M PB at 75 °C to a C_0t of 10,000. Mixtures were diluted 20-fold with ice cold water (to a concentration of 0.012 M PB) and divided into 12 samples. The samples were heated from 45 to 85 °C in 5° increments. After 5 min at each temperature, one sample was removed, chilled, and treated at 50 °C for 40 min with S1 as previously described³. a, Rat ³H-cDNA (Δ) and rat ³²P-scDNA ($\square$) reassociated to rat DNA; b, ³H-cDNA (Δ) and rat ³²P-scDNA ($\blacksquare$) reassociated to mouse DNA; c, As panel a with results expressed as % of total counts hybridised; d, As panel b with results expressed as % of total counts hybridised. The arrows in panels c and d indicate the T_m .

hybridisation, however, measures the complexity of the RNA population and not the radioactive tracer; it is therefore possible that the cDNA probe is of relatively low complexity and homologous to only a fraction of the poly(A)-containing sequences. Verma *et al.*¹⁵ have measured the ability of cDNA to render its template, *Dictyostelium* poly(A)-containing RNA, double stranded in conditions of cDNA excess. This cDNA population seemed to contain all of the sequences present in the poly(A)-containing RNA population. Until a comparable experiment has been performed in this system, the complexity of this cDNA probe is somewhat uncertain. There is, however, little information to suggest selective transcription of specific poly(A)-containing sequences and it is therefore likely that this cDNA probe is a complex mixture of 6,000–7,000 different sequences.

It is also possible that these rat myoblast poly(A)-containing sequences represent only a limited fraction of the total genetic information required during the lifetime of the entire organism and therefore, our conclusions are limited to such a set of sequences. In addition, there might be some mRNA lacking poly(A) which would be undetected in the present study¹⁶. Nevertheless, we feel that this number is large enough to represent an average population of mRNA, and although proteins evolve at vastly different rates, it is expected that a population of 7,000 different proteins will reflect the average properties of the total complement of proteins of which it is a subset.

The cDNA used in this experiment was, on average, 370 nucleotides long, and should represent approximately 18% of the length of messenger RNA molecules³, although

it undoubtedly represents the sequence at the 3' end of the messenger molecules more frequently since it is primed by (pT)₁₀ from the poly(A)-containing end. Work by Proudfoot and Brownlee¹⁷, indicates that there exists an identical poly(A)-proximal 3' sequence in both mouse immunoglobulin mRNA and rabbit haemoglobin mRNA. In fact, this 3' sequence is too small to be detectable by hybridisation, which is consistent with the single-copy nature of the cDNA probe. Significant homology between the 3' untranslated regions of different mRNA sequences is therefore unlikely. Nevertheless, sufficient homology might exist between the 3' untranslated portion of the mRNA molecule which codes for the same protein in both rats and mice to influence the experiments presented here. Preliminary experiments to investigate the thermal stability of heterologous mRNA-DNA duplexes argue against this possibility (K.S.G., M.S.C. and M.R., unpublished). In this case, the rat mRNA-mouse DNA duplexes have a T_m only 2 °C below the T_m of the homologous duplex structures. This is the same result obtained in experiments on haemoglobin mRNA and is consistent with the interpretation presented above⁴. These experiments also suggest that the increased ΔT_m observed with rat myoblast cDNA (5–8 °C) as compared with rat myoblast mRNA (2 °C) may be due to the presence of an increased representation of 3' untranslated regions of these mRNA sequences in the cDNA.

These experiments deal exclusively with the evolutionary distance between rats and mice, and, therefore, with evolutionary events which have occurred in these two rodent species during the past 5–10 Myr. It is conceivable, although unlikely, that such conclusions are uniquely applicable to rodents, recent evolutionary events, or both. Nevertheless, these results suggest that most of the DNA in

mammalian genomes is not involved in translation. Those sequences which are translated comprise at most 30% of the unique nucleotide sequences of the genome and are relatively conserved in nucleotide sequence. The remaining sequences accept nucleotide substitutions more readily, as indicated by the greater divergence of scDNA as compared with cDNA.

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letters to nature

Size distribution of cosmic gamma-ray bursts

WE report on the combined results of three balloon-borne searches for small cosmic γ -ray bursts, carried out by the Imperial College Space Physics Groups. We suggest that our results are incompatible with the extragalactic source postulated for the large events previously seen by satellites. No decisive directional information has been obtained from the timing measurements, carried out by the Vela satellites on the large (10–100 photon $\text{cm}^{-2} \text{s}^{-1}$ ($> 100 \text{ keV}$)) events, although a weak correlation with the local spiral arm exists¹.

Description of the apparatus and results of our first flight have been reported², but a significant increase in viewing time has been achieved. Briefly, a flight of 4.5 h at 5 mbar was achieved from Aire Sur L'Adour on September 12, 1974, with a detector consisting of 10^4 cm^2 of plastic scintillator, 5 cm thick and sensitive to γ rays in the range 15 keV to 1 MeV. One unconfirmed event was seen of size $1.2 \times 10^{-7} \text{ erg cm}^{-2}$ and duration 5 s. Two further flights from Gap (France) were made on June 11 and 21, 1975, with the same plastic scintillator, split into two slabs, each inclined at 30° to the horizontal to gain some directional information. In 9.0 h at an altitude of less than 5 mbar no candidate events were detected. The event search was carried out using various integration times up to 8 s. It is interesting that of the seven large bursts seen by the Vela network whose time profiles are

well determined from other satellite data, six produced most of their energy within 8 s of their peak intensity.

If the sources of γ -ray bursts have a well behaved distribution about a mean strength, and are uniformly distributed in space, it is a consequence of the inverse square law that the observed burst size S in erg cm^{-2} integrated over the event duration and the rate of events $N(S) > S$ are related by $N(S) \propto S^{-\alpha}$ where $\alpha = 3/2$. Alternatively, if the sources are confined to an infinite thin sheet approximating the shape of the galactic disk then $\alpha = 1$. Although this does not require sources to have a uniform intrinsic luminosity and time behaviour, it does depend on the width of the intrinsic source distribution being relatively small compared with the range of sizes measured experimentally, and also on the event duration being small compared with the occurrence rate. It is important to compare the satellite and balloon rates, spanning nearly three decades in size to investigate if either of these predicted distributions fits the data.

A total of 23 Vela bursts, as confirmed by multi-satellite recordings, have accurately known sizes S . Strong *et al.*¹ have already plotted a size spectrum for these events in terms of erg cm^{-2} and shown a fall off from the $-3/2$ law at low intensities. This, however, may be due to a lack of sensitivity in the triggering system used in their experiment³. Figure 1 shows the size spectrum as plotted by Strong together with the Imperial College data, adjusted to a rate from $4\pi \text{ sr}$ and for the same period as used by Strong. In plotting the balloon data, we assume the standard $\exp(-E/E_0)$ spectrum with $E_0 = 150 \text{ keV}$. Note that if the '3/2' power law holds we are

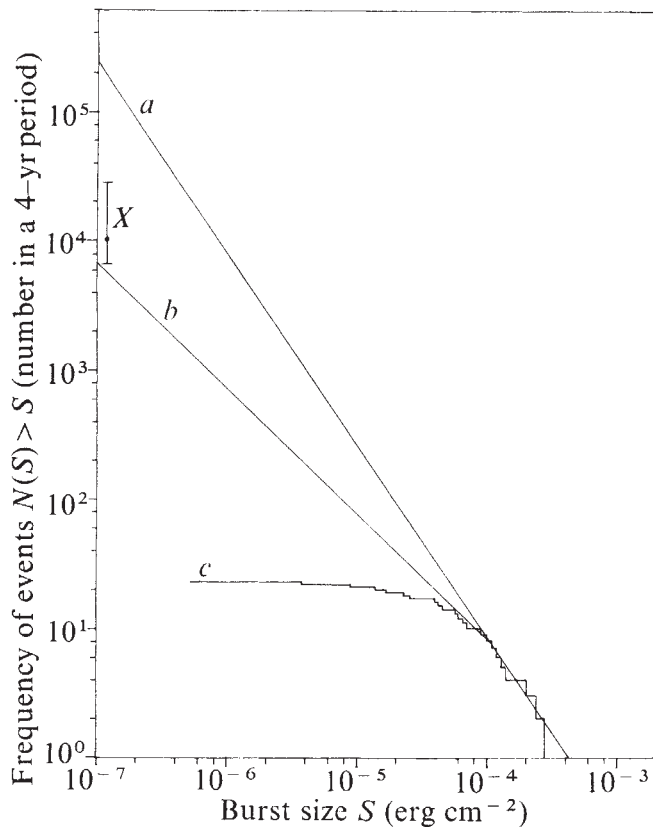


Fig. 1 Plot of burst size (S) against frequency. Our point (X) is marked with $\pm 1\sigma$ error bars calculated from a Poisson distribution. The smooth curves (a and b) give the theoretical $S^{-3/2}$ and S^{-1} distributions extrapolated from Vela results (curve c).

entitled to normalise at any point on the experimental Vela spectrum. The balloon point can be seen to lie close to the S^{-1} extrapolation of the Vela point and the probability of its being a statistical fluctuation from the $S^{-3/2}$ law is negligible. The magnitude of random errors in the size determination from the Vela point is given by the departure of the points from the smooth curve, which approximates the true burst occurrence rate. Any systematic errors in the satellite data, such as missing part of a burst due to the triggering system, will cause the Vela size spectrum to move towards higher sizes, thereby increasing the discrepancy from the '3/2' power law. Similarly, sources of error in the Imperial College result, namely the unconfirmed nature of the event, uncertainties in the angular window of the detector and a possible lower effective energy threshold, can only move the point further from the $-3/2$ line. We have also investigated the size spectra from the two detector types as a function of photon intensity, defining an effective pulse time and mean height, and get essentially the same discrepancy from the $-3/2$ power law. Thus we believe that the conclusion is insensitive to the exact manner in which source strength is defined and to the assumption concerning the spectral form of the candidate balloon event. We can therefore be reasonably confident in excluding a uniform source distribution throughout space (with a relatively narrow intrinsic source distribution), but cannot confirm a galactic disk distribution. Indeed, if the balloon point is not genuine, then the only real events may be large local happenings, all of which we see. One possibility that neither the balloon nor Vela data can adequately investigate, is that an appreciable fraction of burst events occur over time scales larger than 8 s, with slow rise and fall times, and a small peak intensity-time scale ratio compared with those events presently known³. It is possible to imagine an absolute size dependence for this ratio which would invalidate any simple conclusions concerning deviations from a $-3/2$ power law. A more implausible alternative may be

that we are seeing most events from a universal distribution with a rather flat size distribution extending over nearly four orders of magnitude.

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Cosmic ray streaming

TAKING the confinement mechanism for cosmic rays in the Galaxy to be resonant scattering by Alfvén or hydromagnetic waves generated by the streaming of the cosmic rays themselves^{1,2}, I shall show that the abundant low energy cosmic rays can generate, indirectly, enough Alfvén waves of long wavelength to scatter cosmic rays of considerably higher energy. Cosmic rays of energy up to around 1,000 GeV may thus be confined within the Galaxy more efficiently than has previously been supposed.

In highly ionised regions of interstellar space, it is thought that the Alfvén waves so generated, which propagate 'forwards' along the ambient magnetic field B_0 in the direction of decreasing cosmic ray density, are removed by a cascade of stimulated decay processes involving reversal of the Alfvén wave direction and emission of sound waves³. Schematically,

$$A_+ \rightarrow A_- + S \text{ followed by } A_- \rightarrow A_+ + S^*,$$

where $+$ ($-$) refers to a forward (backward) Alfvén photon. At each wave-wave interaction, the Alfvén photon loses some energy to a (compressional) sound wave, which can be damped by thermal conductivity. (Note that the resonant wavelengths are sufficiently long, for cosmic rays of interest, that the plasma is collisional.)

Full details of the analysis are being presented elsewhere⁴⁻⁶, but as long as the sound waves remain heavily damped the steady-state equations governing this cascade are

$$p \frac{\partial \mathcal{J}}{\partial p} = \frac{\alpha_0 \pi v_A p^5}{3 U_M} \frac{\partial f}{\partial p} \quad (1)$$

$$p \frac{\partial \mathcal{A}}{\partial p} = \frac{-4 \alpha_0 p^5 v f}{3 m \Omega_0 U_M \mathcal{J}^2 L} \quad (2)$$

(equations 11 and 12 of ref. 6). Here $\mathcal{J} = \mathcal{J}_+ + \mathcal{J}_-$ = total Alfvén wave intensity per unit log bandwidth (relative to $U_M = B_0^2/2\mu_0$), $\mathcal{A} = (\mathcal{J}_+ - \mathcal{J}_-)/(\mathcal{J}_+ + \mathcal{J}_-)$ is the wave asymmetry, α_0 is a numerical constant relating wavenumber k and particle momentum p in an approximate resonance condition $\alpha_0 k p = m \Omega_0$ (for the galactic particle spectrum, $\alpha_0 = 0.41$) and $L = f B_0/|B_0 \cdot \nabla f|$ is the cosmic ray scale length along field lines. For the escape of cosmic rays from the Galaxy, we shall assume that L is at least approximately independent of energy. In deriving (1) and (2) the wave-wave interaction formulae³ have been coupled with the linear growth or decay of waves due to cosmic rays, expressed in terms of the gradients of the cosmic ray distribution function f .

These two equations determine the Alfvén wave intensities appropriate to a given spectrum of cosmic rays. They do not necessarily imply that the intensities fall off with increasing

p , as f does. Being differential equations, they should be solved with the aid of two boundary conditions. One such boundary condition is that $\mathcal{S}_-$ should vanish ($\mathcal{A} = 1$) at the 'top' of the cascade (largest k , smallest p). This is because the photon flux down the cascade (which varies as the product $\mathcal{S}_+ \mathcal{S}_-$ because it arises from the decay of A_+ stimulated by A_-) must vanish at that k above which there is no further input of photons.

A second boundary condition can be set by the breakdown of the cascade equations at small k (large p). When the wavelengths become large, the sound waves are no longer heavily damped and their intensity rises. A highly asymmetric distribution of Alfvén waves ($\mathcal{A} \simeq 1$) cannot be maintained in the presence of an appreciable level of sound waves, and in fact the cascade equations break down when the dimensionless sound damping coefficient $\mathcal{D} = 4\sigma_s/\pi k v_A \mathcal{S}$ falls to the value v_A^2/v_s^2 (ref. 5). The asymmetry $\mathcal{A}$ is unable to remain close to unity for yet smaller values of k .

On the other hand, $\mathcal{A}$ cannot decrease below (say) $1/2$ before the cascade equations break down, because the ever faster rate of decrease of $\mathcal{A}$ (see equation 2 with $f \propto p^{-4.5}$) as k diminishes would soon give a solution with $\mathcal{A} < -1$, which is clearly impossible. Thus $\mathcal{A}$ must pass through some value like $1/2$ just when $\mathcal{D}$ falls to v_A^2/v_s^2 . In fact, the solutions are fairly insensitive to this condition. The two boundary conditions are

$$\mathcal{A} = 1 \text{ at } p = p_{\min} \quad (3)$$

$$\mathcal{A} \simeq 1/2 \text{ when } \mathcal{D} = v_A^2/v_s^2 \quad (4)$$

Taking as typical parameters for the ionised interstellar medium $|\mathbf{B}_0| = 3 \mu\text{gauss}$, $n_e = 10^6 \text{ m}^{-3}$, $T = 7,000 \text{ K}$ and taking $L = 500 \text{ pc}$, the solution of (1) and (2) under (3) and (4) has the total wave intensity dominated by its constant of integration

$$\mathcal{S} \simeq \mathcal{S}_0 = 4 \times 10^{-4} \quad (5)$$

The corresponding asymmetry obeys $1 - \mathcal{A} \ll 1$ until the bottom of the cascade is approached. (The way in which the results scale with interstellar parameters is to be found in ref. 6.) Even at the lowest particle energies of $\sim 1 \text{ GeV}$, $\mathcal{S}_0$ exceeds the $O(p^4 v_A f/U_M)$ term directly contributed to $\mathcal{S}$ by the right of equation (1). Formally, this solution holds up to a particle energy

$$(p/mc)_{\max} = 7 \times 10^6, \text{ or } E = 7 \times 10^6 \text{ GeV} \quad (6)$$

determined by equation (4). It is, however, possible that general interstellar turbulence may cause even more energy to be given to those waves which resonate with such high energy particles^{7,8}.

The relatively large wave energy (5) enables cosmic rays to be confined very efficiently, with only a small diffusion coefficient. The waves are, however, nearly all forward waves. The net effect of diffusion through a system of waves moving in one direction at the Alfvén speed is to give particles of a given energy a net anisotropy

$$a = (1.5 v_A + (D_{\parallel}/L))/v$$

where 1.5 is the Compton-Getting factor and the diffusion coefficient is $D_{\parallel} = 4pv/3\pi m\Omega_0 \mathcal{S}_0$. Thus the predicted anisotropy at a typical location within the galactic disk is, numerically

$$a = (1.0 + (E/180 \text{ GeV})) \times 10^{-4} \text{ for } L = 500 \text{ pc} \quad (7)$$

The corresponding anisotropy for a shorter assumed scale length is

$$a = (1.0 + (E/100 \text{ GeV})) \times 10^{-4} \text{ for } L = 200 \text{ pc} \quad (8)$$

Even 1,000 GeV particles have an anisotropy less than 0.1%, in keeping with observational constraints up to that energy.

To conclude, the intensity of self-generated Alfvén waves may be much higher than the $O(p^4 v_A f/U_M)$ which one would suppose for power law solutions of the wave equations, in particular equation (1). Instead, the constant of integration dominates, and this gives a 'flat' spectrum having equal energy in each octave of waves. These high intensity waves give good confinement of cosmic rays having $E \lesssim 1,000 \text{ GeV}$. Above this energy, though, it may be necessary to appeal to an interstellar turbulence spectrum for a source of hydromagnetic waves.

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Third speed-up of the Crab pulsar

OPTICAL timing of the pulsar in the Crab nebula revealed a further speed-up by $\Delta v = 0.097$ periods $\text{d}^{-1} = 3.7 \times 10^{-8} v$, on 1975 February 4 (JD 2442447.9). The initial jump in pulse frequency was at least three times larger than that reported¹ for the first glitch, and allows a better estimate of the glitch parameters.

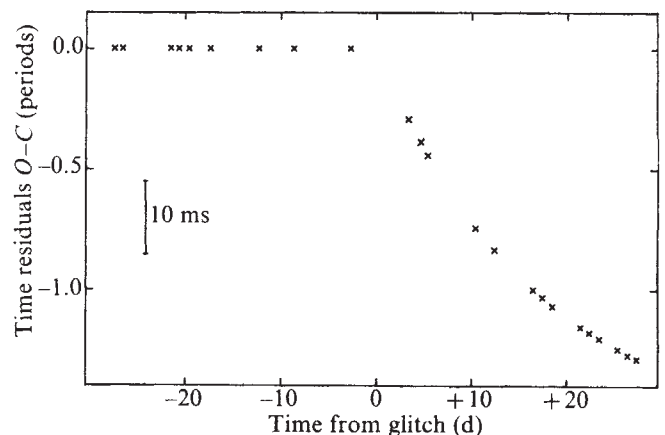
Arrival times of optical pulses from the Crab pulsar were measured at the 60-cm refractor of the Hamburg-Bergedorf Observatory. The typical error for one night is about $10 \mu\text{s} = 0.0003$ periods relative to Loran-C Silt signals. The barycentric arrival times were computed using a program by J. M. Rankins² and an ephemeris tape prepared by the MIT Lincoln Laboratory³.

Figure 1 shows time residuals (observations—calculations) from a cubic fit to the barycentric arrival times before the glitch according to:

$$O - C = \phi_0 + (t - t_0) v_0 + (t - t_0)^2 \dot{v}_0/2 + (t - t_0)^3 \ddot{v}_0/6 - N$$

Only ϕ_0 , v_0 , and $\dot{v}_0$ have been fitted: $\ddot{v}_0$ was fixed, because short time spans usually yield meaningless values of $\ddot{v}_0$ (see ref. 4 and Fig. 1 of ref. 5). The value adopted, $\dot{v}_0 = 7.95 \times 10^{-6} \text{ d}^{-3}$

Fig. 1 Observed pulse arrival times minus arrival times computed from cubic fit to the data before the third glitch.



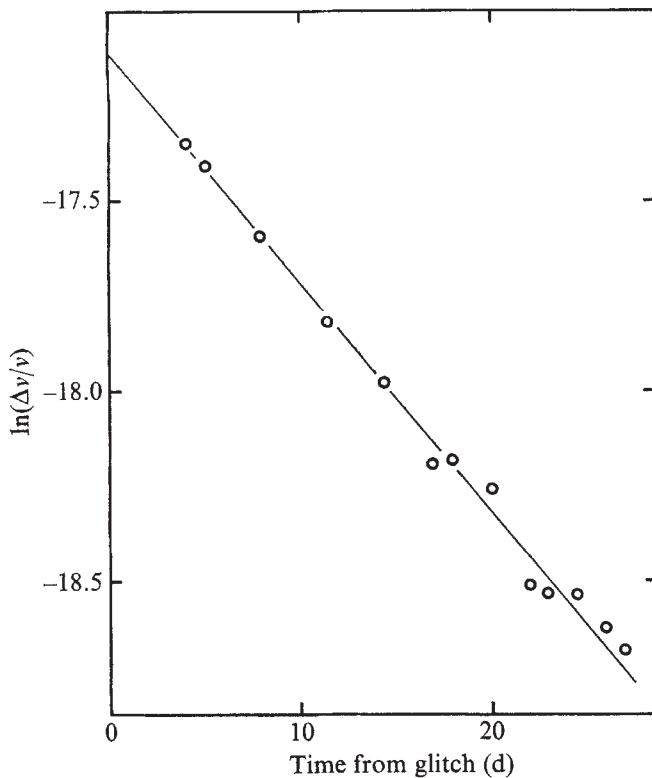


Fig. 2 Natural logarithms of excessive pulse frequency.

corresponds to a braking index n ($\equiv v\ddot{v}/\dot{v}^2 \approx$) 2.523, which is very close to the mean value⁶ over the five years following the first speed-up. The observational errors and the deviations from the fit are too small to be seen on the scale of Fig. 1. A sharp increase in the pulse frequency, resulting in an initial phase shift of 0.1 period d^{-1} appears after February 4. The frequency shift slowly decays, approximately following an exponential law.

In Fig. 2 the natural logarithms of the frequency differences Δv ($v_0 - v_c$) from the cubic fit are plotted. Here Δv has been taken as the ratio of the differences in $O-C$ divided by the differences in time for each pair of adjacent observations. The error bars include observational errors only. A straight line drawn through the points of Fig. 2 yields a relaxation time of 17 d for the frequency jump.

A two-component model, proposed by Ruderman⁷ and Baym *et al.*⁸ has been applied to the first glitch of the Crab pulsar by Boynton *et al.*¹. According to this model the residuals $O-C$ from a cubic fit are

$$O-C = -\Delta v_0 \{ Q\tau(1 - \exp[-(t-t_g)/\tau]) + (1-Q)(t-t_g) \}$$

where Δv_0 is the initial frequency jump, τ the relaxation time, t_g the time when the glitch occurred, and Q a structure parameter equal to the ratio of the moments of inertia of the superfluid component to the moment of inertia of the whole star.

The parameters of the three glitches known at present are compiled in Table 1, where data for the first glitch are from ref. 1. The errors are twice the formal errors of the fit. The second⁹ speed-up was too small to give definite values of Q and τ at the same time, therefore Q has been fixed and the post-glitch span has been restricted to 14 d; longer fits with free Q yield $Q \sim 0.65$ and $\tau \sim 5$ d. It might be possible to gain useful information by combining this small glitch with events of similar size to reduce the frequency noise. At the moment it is not clear what causes the discrepancy between the relaxation times of the first and the third event, but two sources of errors appear in the pre-glitch data of ref. 1: first the large residuals displayed during the summer months—possibly the consequence of an earlier large glitch—make it difficult to estimate $\dot{v}$ and $\ddot{v}$ from a longer

interval, and second, the large gaps in the two shortest fits make it impossible to determine $\dot{v}$ and $\ddot{v}$ reliably. Keeping in mind these difficulties it seems unjustified at present to propose essentially different relaxation times for the first and the third glitch.

Because of the frequent small jumps in period, all parameters derived from fits of timing observations depend on the length of the fit, the weighting, and the distribution of the data points. In the data of the third glitch, however, this effect is not serious for short spans. Even the shortest fit, extending to 12.5 d after the glitch only, yields parameters which agree well with those in Table 1, namely $\Delta v_0 = 0.097 \pm 0.006 d^{-1}$, $Q = 1.04 \pm 0.56$, $t_g = JD\ 2442447.96 \pm 0.15$, and $\tau = 16.5 \pm 14$ d (formal mean errors), where the large errors in Q and τ may be explained by the high correlation between these two parameters. If we go to larger intervals, the parameters stay within the limits of Table 1 when two further points, -42 and $+33$ d from the

Table 1 Parameters for three Crab pulsar glitches

Date	1969 September 29	1971 October 26	1975 February 4
Julian day			
-2440000.5	0493.8 ± 0.6	$1,250.3 \pm 0.3$	$2,447.4 \pm 0.1$
$\Delta v_0 (d^{-1})$	0.025 ± 0.009	0.0052 ± 0.0006	0.097 ± 0.002
$\Delta v_0/v (\times 10^6)$	9.5 ± 3.5	2.0 ± 0.2	37.2 ± 0.8
$\tau (d)$	4.1 ± 2.2	15.2 ± 4.0	15.5 ± 1.2
Q	0.93 ± 0.05	0.96 (fixed)	0.96 ± 0.03
Span of fit			
pre-glitch (d)	37	22	27
post-glitch (d)	25	17	28

glitch are included. If we include points at 47 d and more after the glitch, Q exceeds 1 and τ becomes greater than 20 d. It is possible, however, to keep Q and τ within the limits of Table 1 if we change $\dot{v}$ by $\sim 20\%$, which is within the range experienced when fitting polynomials to spans of comparable length during 'quiet' periods.

So, at present there is no need to assume a significant deviation from the two-component model. It may be hoped that a somewhat better value for Q will be obtained from the better coverage of this last event, especially from timing data of the month preceding and the two weeks following the range of Fig. 1.

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High temperature condensates among meteors

HARVEY has reported¹ the spectra of two meteors which exhibited no lines of iron or sodium, but which did show lines of calcium and magnesium as well as of aluminium and silicon. Meteors characterised by this type of spectrum constitute about 1% of the 500 meteors observed by the NASA-LRC Faint Meteor Spectra Patrol². Harvey recognised that the spectra indicated chemically differentiated material, and suggested that the

Table 1 Average Na and Fe contents of meteorites* and the lunar surface

Sample	Class	Na(10^{-6} g g $^{-1}$)	Fe (%wt)
Carbonaceous chondrites	C1	5,100	18.4
	C2	3,800	21.9
	C3	3,500	25.2
Ordinary chondrites	H	5,700	27.6
	L	6,500	21.8
	LL	6,600	20.0
Enstatite chondrites	E4	8,200	33.0
	E5,6	5,200	25.5
Ca-poor achondrites	Aubrites	3,500	1.0
	Ureilites	300	14.4
	Diogenites	120	13.5
Ca-rich achondrites	(ref. 13)		
	Chassignite		20.6
	Eucrites	2,800	13.9
	Howardites	1,900	14.4
	Nakhlites	3,300	16.6
Lunar surface	Shergottite	11,000	
	Angrite		7.5
		1,430–5,000	6.5
		(ref. 14)	(ref. 15)

*Except as noted all data are from ref. 12.

meteoroids were composed of enstatite achondritic meteorites (aubrites) to explain their lack of iron.

Obtaining elemental abundances from meteor spectra is difficult. Since the Na D lines are, however, among the most intense of the persistent lines of the elements, the absence of Na from the spectra merits consideration. In Table 1 are listed Na and Fe compositions of various well studied Solar System materials, including aubrites. It can be seen that none simultaneously shows low iron with low Na. The Na content of aubrites, which is similar to that of carbonaceous chondrites, is not unusually low. Observations² and laboratory experiments³ indicate that most meteors are produced from undifferentiated material having non-atmophile elements present in amounts similar to those in carbonaceous chondritic meteorites. Sodium is always a feature of their spectra. Thus, it seems necessary to consider possible parent materials other than aubrites to produce the observed iron- and sodium-free meteors.

In searching for another source, we started with the minerals thought to be of cosmological interest which contain both Ca and Mg but neither Fe nor Na, namely, melilite, ($\text{Ca}_2\text{Al}_2\text{SiO}_7$ – $\text{Ca}_2\text{MgSi}_2\text{O}_7$) and diopside ($\text{CaMgSi}_2\text{O}_6$). These minerals are among those predicted by Grossman⁴ to be early (or refractory element) condensates of the primitive solar nebula, that is, chemical compounds which would condense from a cooling gas of solar composition at a total pressure of 10^{-3} bar and temperatures exceeding 1,400 K. This suite of rather unusual minerals has received wide attention because the minerals have been identified in light-coloured, millimetre-sized polymineral inclusions present in carbonaceous chondrites (reviewed in ref. 4). It should be noted that almost the entire suite can be formed from Mg, Ca, Al and Si, the two exceptions being the Fe–Ni alloy and Ti-bearing perovskite (CaTiO_3). Metallic Ni–Fe is not found in association with the other members of the suite in carbonaceous chondrites⁵, so the absence of Fe from the meteors is not surprising. The other minerals in the suite are corundum (Al_2O_3), spinel (MgAl_2O_4), and forsterite (Mg_2SiO_4). By reason of its refractory nature, Ti should also be present in these meteoroids. It might, however, not have been detected in the meteor spectra because of the experimental difficulties reported by Harvey³ for other refractory elements, and because of its lower abundance, 3×10^{-3} times the cosmic abundance of Al.

There is at least one problem in identifying the refractory inclusions found in meteorites with the Mg, Ca-rich meteors, namely, some inclusions in the carbonaceous chondrite Allende have rather high Na contents (up to 25,000 p.p.m.⁶). The Na seems, however, to be located in the inclusions in separate mineral phases, such as sodalite ($\text{Na}_8(\text{Al}_6\text{Si}_6\text{O}_{24})\text{Cl}_2$) and

nepheline ($\text{NaAlSi}_3\text{O}_8$) and there is evidence that the alkali-bearing minerals were formed by chemical reactions which occurred after the inclusions were emplaced in the carbonaceous chondritic matrix^{4,6–8}. It should be mentioned that the refractory minerals, as opposed to the alkali-bearing minerals, in the inclusions are free of Na and Fe⁹.

Because of the ubiquity of Fe and Na in planetary materials, it is necessary to eliminate all known types of meteorites from consideration. Since most stony meteorites, especially the chondrites, are complex mixtures of materials, which may have had differing origins and histories, it is reasonable to suppose that the components of meteorites such as the refractory inclusions, may be found individually in space. The refractory inclusions found in carbonaceous chondrites have in all cases been small objects typically less than a centimetre in diameter. Their small size and undistinguished appearance may explain why none has been found as an individual meteorite on Earth.

On the basis of spectral indicators, Harvey¹ assigned the Ca, Mg-rich meteors high velocities ($V > 40 \text{ km s}^{-1}$) and, hence, high inclination and/or retrograde orbits. Such orbits are generally associated with comets, although some asteroids are also found in highly inclined orbits. Comets are believed to be the most volatile-rich objects entering the inner Solar System, whereas the refractory minerals are the least volatile substances known. Such a vast compositional difference would seem to preclude a genetic relationship, other than mixing at low temperatures, between the two, in spite of their similar orbits.

The interesting possibility arises that these meteoroids could form a reservoir for the so-called 'lost' elements: Ca, Mg, Al, Ti, the lanthanides, and other refractory elements which are depleted in ordinary and enstatite chondrites relative to cosmic abundances¹¹. This suggestion would seem to require that early condensates from the solar nebula were separated from later condensates by some process which resulted in long period, comet-like orbits for the refractory materials. More studies are needed to determine whether or not high entry velocities are indeed characteristic of these meteors. Further compositional data on meteors should help to clarify the nature of the interrelationships among individual meteoritic components, asteroids and comets.

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Detection of a potassium cloud near Io

JUPITER'S innermost Galilean satellite Io is surrounded by an extensive cloud of sodium and hydrogen^{1,2}. The hydrogen is observed to extend along a segment of Io's orbit but the sodium cloud envelopes the entire Jovian system, extending tens of arc min from Jupiter. It is brightest, however, near Io and in its orbital plane. Sputtering by

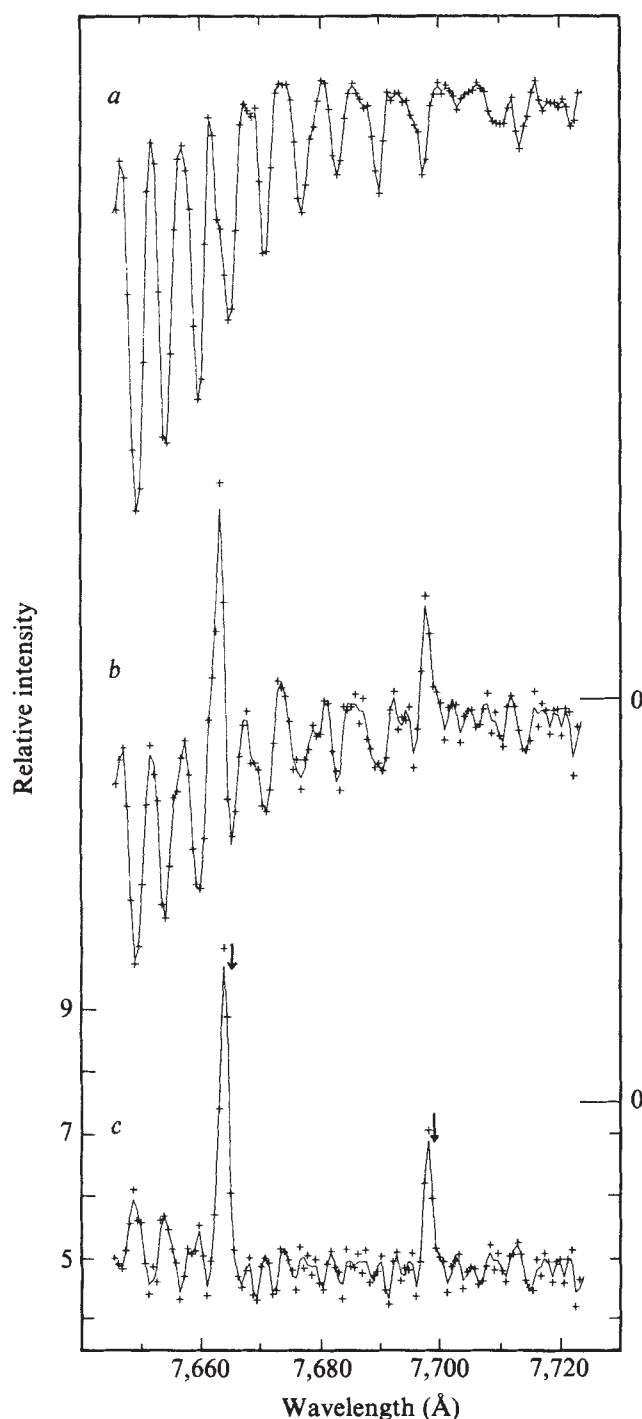


Fig. 1 Potassium emission in Io's cloud. These spectra were taken on September 5, 1975 using the coude scanner of the 107-inch telescope at the McDonald Observatory. The resolution is 1.3 Å. The original data points are shown superposed on the slightly smoothed spectra. *a*, Spectrum of Io in the region of the potassium resonance lines showing absorption from the strong telluric O₂ band lying between 7,594 Å and 7,700 Å. *b*, Spectrum of Io's cloud 7½'' east of Io showing the potassium lines at 7,665 Å and 7,699 Å in emission. Io was east of Jupiter at this time, with an orbital phase angle of 64° from superior geocentric conjunction. The spectrographic parameters were the same as those for *a* except the entrance slit measured 3.0'' × 22.5'' instead of 3.0'' × 4.2''. Io's diameter at this time was 1.21''. *c*, the ratio spectrum. The telluric absorption is nearly cancelled out. The arrows mark the wavelengths of these lines in the Earth's rest frame. The emission lines are blueshifted 1.1 ± 0.3 channels, a result consistent with the 1.3 channels expected from Io's Doppler shift of -0.84 Å relative to the Earth.

charged particles impacting the surface of Io has been the most plausible hypothesis for supplying the sodium³. The hydrogen may result from neutralisation of protons trapped in the Jovian magnetosphere when they impact on Io's surface⁴. Resonant scattering seems to be the dominant emission mechanism^{5,6}.

Sputtering suggests that other elements in Io's surface layer should also populate the cloud. Various searches for such elements, however, have not proved successful^{1,7,8}. Even when Io's disk was excluded from the spectrograph slit, no emission other than from sodium was obvious⁹. I report the first detection of potassium in Io's cloud. Its presence with hydrogen and sodium, along with the absence of more abundant elements such as calcium, might be explained by a rough surface microstructure for Io and a process for multiple impacts of the sputtered products with the surface.

Figure 1*a* and *b* shows a spectrum of Io and of a point 7½'' east of Io in the telluric oxygen band at 7,600 Å. Figure 1*c* shows the ratio spectrum and the emission lines free of the telluric absorption. The emission features lie at the wavelengths of the potassium resonance lines 7,665 Å and 7,699 Å in Io's Doppler shifted reference frame. The relative intensity of the two lines is compatible with that of their oscillator strengths, as expected for an optically thin medium. The absolute intensity is not great enough for these lines to be visible when Io's bright disk lies in the spectrographic slit.

Spectra taken 1° north of Io immediately afterwards showed no hint of potassium emission. A spectrum taken 15'' east of Io showed the emission at about half the strength. A final spectrum at 7½'' east of Io, 1 h after that given in Fig. 1*b* showed the emission strength to be essentially unchanged. Thus, the emission is associated with Io and lies, like sodium, in an extensive cloud about Io.

Since only elements with unit valence have been detected in Io's cloud, there is a process or condition which favours these elements relative to the cosmically more abundant ones. One interpretation is that Io's surface is enriched in the observed elements relative to solar, lunar or meteoritic abundances. Fanale *et al.*¹⁰ have advanced an evaporite salt hypothesis which has been used to explain this enrichment. They suggest Io's surface is rich in the salt residues of solutions which migrated to the surface and evaporated. Their hypothesis predicts that although the calcium abundance is low, magnesium and sulphur should be enhanced on Io's surface. The lines of these elements must be measured from outside the Earth's atmosphere. The implications of the detection of potassium for this hypothesis are not yet clear.

I point to an alternative interpretation of the preponderance of alkali metals in Io's cloud, which enables Io's surface to have a more typical composition. Laboratory experiments by Cassidy and Hapke¹¹ show that sputtered sodium and potassium are hardly adsorbed on surfaces they impact. On the other hand, other elements are selectively adsorbed in direct proportion to their atomic weight. Cassidy and Hapke suggest that Io's surface microstructure may be rough enough for multiple scattering of the sputtered products to occur, thereby leading to a cloud rich in sodium and poor in other elements. My detection of potassium reinforces this suggestion. Sodium and potassium may occupy a larger portion of the cloud than of Io's surface material.

A difficulty with this suggestion is that a significant portion of the sputtered products will be directed away from the surface so that they cannot directly suffer multiple scattering. This would result in inadequate enhancement of sodium relative to calcium. For selective removal of other elements to occur with high efficiency, it seems necessary for a process to exist which causes upward sputtered elements to reimpact the surface rather than to

escape directly from Io's atmosphere. Such a process might be the effect of a plasma sheath¹² on ionised sputtered products, aided by particle scattering in Io's atmosphere.

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Angular momentum transfer to the inner Jovian satellites

THE transfer of angular momentum from a central rotating body in the presence of a magnetic field has been discussed in connection with the evolutionary history of the Solar System¹. We here consider angular momentum transfer in the inner Jovian satellite system. Electron flux measurements near Io's flux tube², and theoretical estimates of the electric current flowing through Io's flux tube³ are used to estimate the angular momentum transfer during the evolutionary history of the Jovian system. We find that, under certain conditions, those currents are sufficient to produce an angular momentum transfer from Jupiter equal to the present angular momentum of the inner satellites.

Consider the inner satellites to be those whose orbital radii are $\lesssim 15r_J$ (Jovian radii). Within this region the planetary magnetic field is orderly and adequately described as a magnetic dipole⁴. Unless the rotational angular velocity is constant throughout the system, potential differences will occur, resulting in current loops^{1,3}, as sketched in Fig. 1. The electric current flow across magnetic field lines results in a force which produces angular momentum transfer¹.

At an earlier stage of evolution, the material out of which a satellite formed is distributed around the parent body near the equatorial plane¹. We assume here, for accuracy, that this material is spread out as a torus of small radius a and large

Fig. 1 A sketch of an electric current loop in the Jovian magnetosphere. The arrows indicate the directions of current flow along and across dipole magnetic field lines.

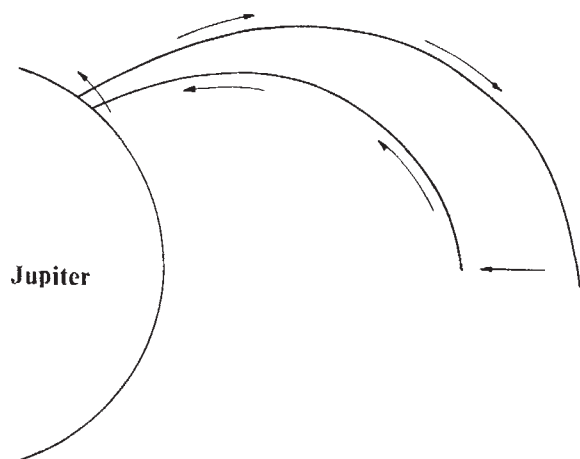


Table 1 Values of a , the small radius of satellite toruses

Satellite	Orbital radius (r_J)*	$a(r_J)$	a/r_J †
Amalthea	2.6	5.0×10^{-4}	0.3
Io	6.0	7.5×10^{-2}	3
Europa	9.5	8.8×10^{-2}	4
Ganymede	15.0	2.2×10^{-1}	5

* r_J , radius of Jupiter (present values⁵).

† r_s , satellite radius.

radius r equal to the present orbital radius of the satellite. The angular momentum transfer ΔL from Jupiter to the torus in time Δt can be expressed as $\Delta L = rJBV\Delta t/c$, where J is the current density flowing through the torus, B is the magnetic field magnitude at r , $V (= 2\pi^2ra^2)$ is the volume of the torus, and c is the velocity of light in a vacuum. The present angular momentum of the satellite is $L = 2\pi mr^2/T$, where m is the mass of the satellite, and T is its orbital period. For significant angular momentum transfer $\Delta L = L$, and

$$a = \left(\frac{mc}{\pi TJB\Delta t} \right)^{1/2}$$

We now proceed to evaluate a for the inner satellites to find out if we arrive at plausible values for this radius. The masses and orbital periods of the satellites are well known⁶. The magnetic field in the magnetic equatorial plane is $B = 4(r_J/r)^3$ gauss (ref. 4). For Δt we use a rough estimate of the evolutionary time scale of the Jovian system, $\Delta t = 10^{16}$ s. The choice of a value for the current density J is based on the following considerations. Io has attracted attention due to the associated decametric radio bursts. It seems probable, however, that current systems are, or have been, present at other Jovian satellites. It has been reported⁶ that Jovian radio bursts seem to be correlated with the phase of Europa, in addition to the well known correlation with Io's phase. Since there are no independent estimates of the magnitudes of the currents at satellites other than Io, we assume here that the current density J for all satellites is the same as that flowing at present along Io's flux tube. We adopt the working value of $J = 3 \times 10^{-7}$ A m⁻² (ref. 3) which is compatible with recent measurements of electron fluxes near Io's flux tube². (These electron measurements yield an upper limit of $\sim 2 \times 10^{-7}$ A m⁻² for the current due to the electrons measured above the detector threshold of 460 keV.)

The resulting values for the small radius a of the evolutionary toruses are given in Table 1, where we observe that they are smaller than the orbital radii, and of the same magnitude as the present satellite radii. We conclude that the process we are considering is plausible.

This calculation suggests that angular momentum transfer via electric current loops could have been a significant process in the evolutionary history of the Jovian system. One of the critical parameters in the calculation is the magnitude of the electric currents. We have not assumed a value for this quantity, but instead we have used estimates of the current density in Io's flux tube which were arrived at independently to explain other physical phenomena, and which are compatible with recent measurements.

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Unusual series of type III-RS solar radio bursts

SOLAR radio emission on August 12, 1975, was unusual because of the large number of, and predominance of, type III-RS bursts ('reverse slope', positive frequency-drift type III bursts). These bursts seem to be second branches of U bursts and, as such, are consistent with only one current model. Four burst events have a Y-like profile and may be the first observations of the reflection of the burst exciter at a magnetic mirror.

Normal type III solar radio bursts drift rapidly toward lower frequencies. The negative drift ($df/dt < 0$) implies that an electron stream is moving upwards in the corona, exciting oscillation of the coronal plasma at progressively lower local electron plasma frequency and harmonics¹. Variants of type III bursts include U bursts and type III-RS bursts. After drifting to a minimum frequency, U bursts turn and drift towards higher frequencies; the electron stream seems to reach a maximum altitude and be deflected back towards lower altitudes, possibly being guided along a magnetic arch¹. Type III-RS bursts are rarely seen and seem to differ from type III bursts only in the sign of their drift rates; the electron stream thus seems to move downwards in the corona.

During the period 0457–1828 UT, August 12, 1975, the Durnten spectrograph (sweeping 125–1,000 MHz every 0.25 s) observed about 30 type III-RS bursts (Figs. 1a and b) and only ~ half that number of type III bursts. All bursts were observed in the approximate range 200–420 MHz, which is a window in local interference. Sensitivity decreases below 200 MHz. In spite of higher sensitivity in the range

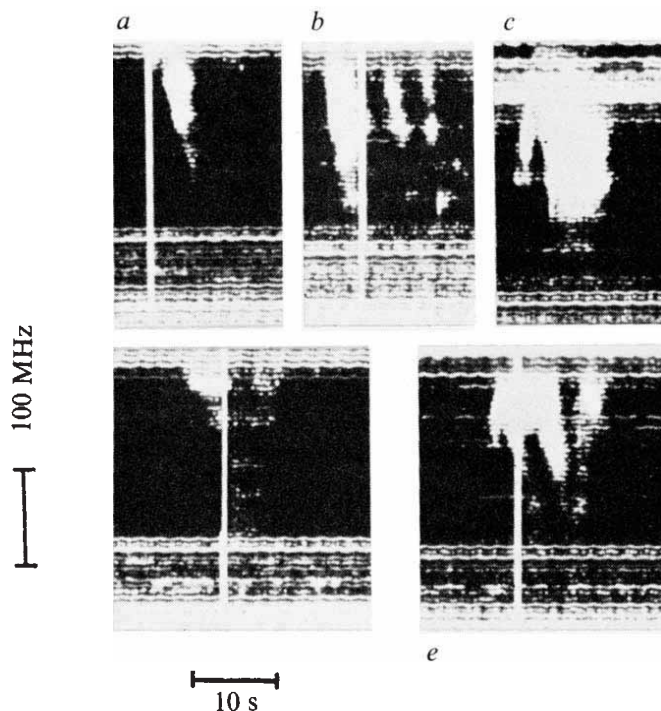


Fig. 1 Dynamic spectra of type III-RS bursts observed August 12, 1975. Frequency increases downwards, frequency ranges of examples are a, b, d, e, 200–500 MHz; c, 140–440 MHz. Times (UT) of examples are: a, 1714; b, 1102; c, 1102; d, 1651; e, 1507. The vertical lines are time markers; horizontal are transmitters.

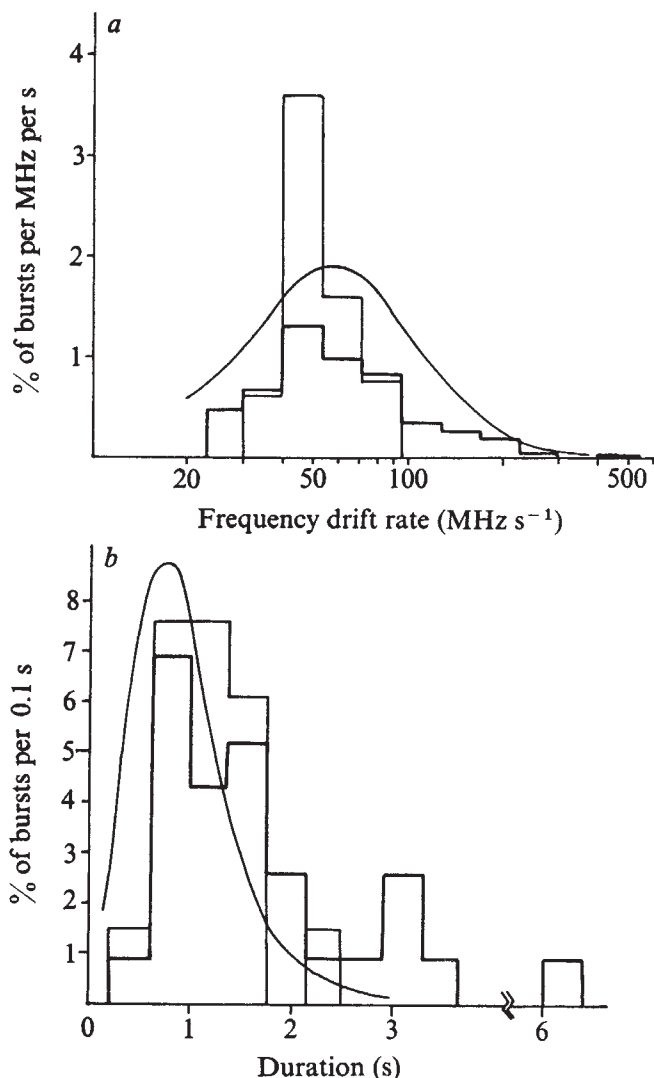


Fig. 2 a, Distributions of frequency drift rates, df/dt , measured in the range 200–420 MHz, for type III-RS bursts (heavy histogram, $N = 30$) and type III bursts (light histogram, $N = 17$) observed August 12, 1975. The curve is a combination of smoothed distributions for type III bursts measured in the ranges 145–175 MHz ($N = 339$) and 310–340 MHz ($N = 245$)⁶. b, Distributions of total duration measured at about 310 MHz for type III-RS bursts (heavy histogram, $N = 30$) and type III bursts (light histogram, $N = 17$) observed August 12, 1975. The curve is a smoothed distribution of type III burst total durations ($N = 230$) measured at 318 MHz (ref. 6).

400–750 MHz, few bursts extended to 500 MHz and none past about 600 MHz. The distributions of drift rate and duration are similar to those of type III bursts (Fig. 2). Two bursts were second branches of U bursts (Fig. 1c and e). Four events contain a type III-RS burst followed immediately by a type III burst, in a Y-like configuration (Fig. 1d and e). We will refer to these bursts as Y bursts. The Y bursts are isolated events and do not appear to be due to chance sequences of bursts of types III-RS and III; in the period 1453–1714 UT the only bursts recorded were three Y bursts.

The Y bursts seem to result from the reflection of a downward travelling electron stream at a magnetic mirror. The mirroring region must, however, be contained in the few times 10^4 km between the burst source and the photosphere; the 10^5 km s^{-1} electrons could reflect and return to the source region in a time of the order of 1 s. About 3 s elapse in Fig. 1e between the disappearance of

the type III-RS burst and that of the type III burst at 300 MHz.

Since type III-RS bursts are rare, we may assume that the bursts in this extended series are homologous, originating in the same general source region and from similar acceleration events. Two of the bursts being second (and stronger) branches of U bursts suggests that all the type III-RS bursts are second branches of U bursts. As simple as this interpretation appears, it disagrees with most U burst observations and models, where the second branch is weaker. If we allow that the situation may be reversed, we must exclude explanations of the weak second branch which are not reversible. Models using a major disturbance to the stream², continuous loss of energy³, and less efficient emission travelling along the density gradient⁴ cannot be reversed. On the other hand, the suggestion (ref. 5, and see ref. 2) that the electron stream becomes less dense due to diverging field lines is reversible; the electron stream entering a region of converging field lines would become denser and a more efficient source of emission and would be likely to mirror.

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Optical detection of Fe³⁺ in lunar plagioclase

NATURAL plagioclase from terrestrial sources exhibits a cathodal luminescence spectrum which consists in general of three emission bands^{1,2}. The emission band in the blue region of the spectrum is thought to be associated with lattice defects³, but the other emission bands are caused by transition-metal impurities present in concentrations of the order of 100-1,000 p.p.m. In particular, the emission band centred at 560 nm has been ascribed to Mn²⁺ ions substituting in Ca²⁺ sites, and the emission band on the edge of the infrared has been shown to be caused by Fe³⁺ ions probably in tetrahedral Al³⁺ sites³. The relative intensities of these emission bands vary from sample to sample, but the Fe³⁺ emission is often dominant in natural terrestrial plagioclases. These conclusions, concerning the nature of the luminescence centres involved, were arrived at by studying the emission characteristics of selectively activated, high purity, synthetic plagioclases³. Optical measurements presented there show that, using excitation spectroscopy, the detection of Fe³⁺ is possible in the plagioclase fraction of lunar fines.

Luminescence spectra of lunar plagioclases measured at room temperature do not show an easily discernible Fe³⁺ band; the presence of such ions in lunar plagioclase is, therefore, generally much less evident than in terrestrial samples of the same mineral. (The same is true of plagioclases of other extraterrestrial origin, such as those from meteorites.) But measurements of emission and excitation

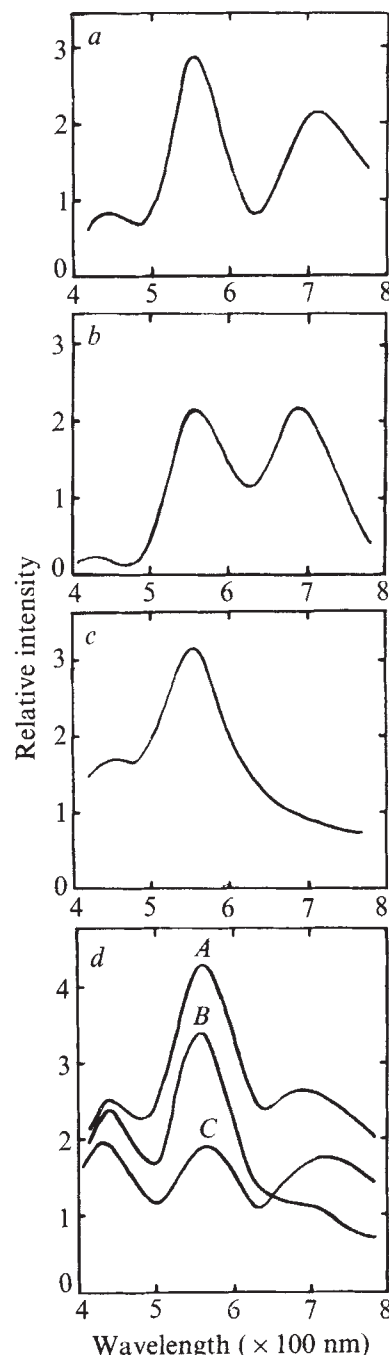


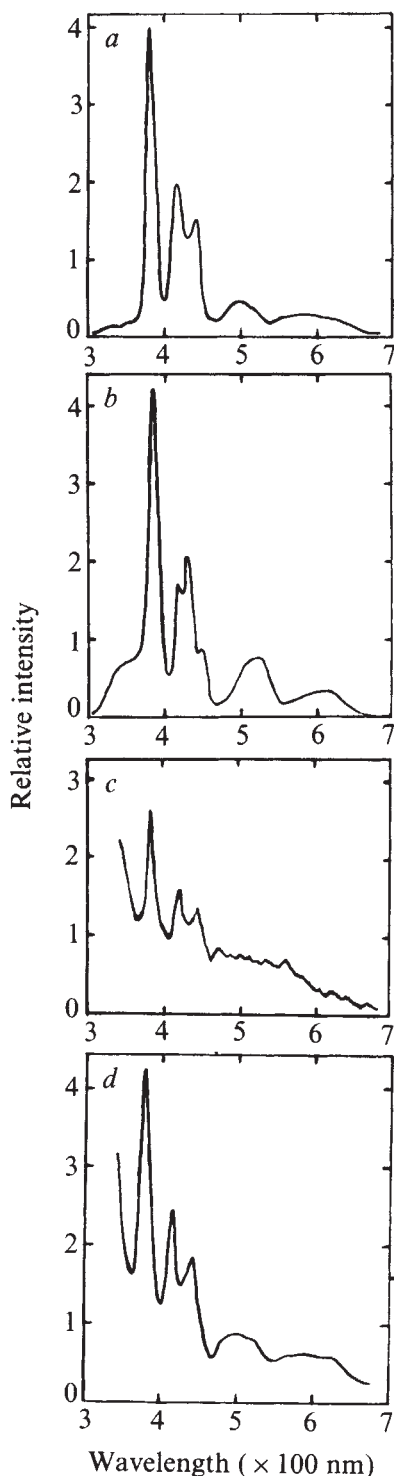
Fig. 1 Luminescence emission spectra under 10 keV electron excitation. *a*, Natural terrestrial bytownite (Emmons Suite No. 25), (see ref. 9); *b*, synthetic anorthite with 600 p.p.m. Mn²⁺ and 4,800 p.p.m. Fe³⁺ added; *c*, unseparated lunar fines 15061.611; *d*, lunar breccia 67455.3.1 (see text for explanation): *A*, 5 K, 8 Hz; *B*, 295 K, 8 Hz; *C*, 5 K, 29 Hz. The relative intensity scales (ordinates) for the different samples are unrelated. All spectra corrected for instrument response and measured at room temperature except where otherwise indicated.

spectra at temperatures down to 4 K have shown that Fe³⁺ is certainly present in lunar and meteoritic plagioclases. Though measurements have so far covered only a few samples, it is likely that these results are typical; Fe³⁺ has been detected in all of the samples investigated. These results are consistent with the reported magnetic detection (electron spin resonance) of Fe³⁺ in a lunar anorthosite (15415)⁴ and in the plagioclase fraction of rock sample 14053.47 and other lunar rocks⁵.

Figure 1 shows the luminescence emission spectra of typical terrestrial, synthetic and lunar plagioclases. There

is no detectable Fe^{3+} emission band in the spectrum of lunar fines 15061 in Fig. 1c. In Fig. 1d curve B is the emission spectrum of the lunar breccia 67455 at room temperature, in which the Fe^{3+} emission band is barely detectable. On cooling to 5 K this band becomes much more pronounced because the intensity of the Fe^{3+} emission has increased much more than the intensity of the green Mn^{2+} emission. Curve C is a time-resolved spectrum

Fig. 2 Luminescence excitation spectra of the Fe^{3+} emission. *a*, Natural terrestrial plagioclase (Emmons Suite No. 25, 77% anorthite)⁹; *b*, synthetic anorthite with 4,800 p.p.m. Fe^{3+} added; *c*, lunar fines 15601.611; *d*, lunar breccia 67455.3.1. Relative intensity scales (ordinates) for different samples are unrelated. A Wratten 70 filter was used to isolate the Fe^{3+} emission. All spectra measured at 5 K.



which uses the fact that the Mn^{2+} emission has a longer lifetime than the Fe^{3+} emission to separate the bands still further. This was achieved by increasing the modulation frequency of the electron beam used for excitation from 8 to 29 Hz.

The spectral position of the Fe^{3+} emission peak varies somewhat from sample to sample, and some correlation with plagioclase composition has been found³.

Though Fe^{3+} can be detected in emission, particularly at low temperatures, the most sensitive and conclusive method of detection is that of luminescence excitation spectroscopy. Using this method the total emission in the deep red or near infrared is isolated by a filter and monitored using a photomultiplier as the sample is excited by intense monochromatic light scanned in wavelength. Figure 2 shows the Fe^{3+} excitation spectra for the same samples for which the emission spectra were presented in Fig. 1. The Fe^{3+} ligand-field bands can be recognised easily in the excitation spectrum of the fines 15601 in Fig. 2c whereas the emission spectrum in Fig. 1c did not show any evidence of the presence of Fe^{3+} . Considering the similarities of the spectra in Fig. 2, there can be little doubt that lunar plagioclases also contain Fe^{3+} , although in lower concentrations than in natural terrestrial samples. These spectra are similar to those of Fe^{3+} in LiAl_2O_3 obtained by Pott and McNicol⁹ and also similar to the absorption spectrum of Fe^{3+} in an iron orthoclase determined by Faye⁷.

Excitation spectra of the Mn^{2+} emission in such plagioclases have also been obtained, thereby providing additional evidence as to the nature of that centre, but these will be presented elsewhere.

Luminescence excitation spectroscopy is obviously a sensitive and effective way of obtaining absorption spectra of luminescent species from powdered or mixed crystalline samples⁸. The method does not require any mineral separation at all, neither does it require highly polished surfaces which are necessary for electron microprobe work.

Experimental details and assignments of the observed ligand-field bands of Fe^{3+} and Mn^{2+} in plagioclase feldspars, including calculations of ligand-field parameters, will be given elsewhere.

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Stratospheric measurements of H_2O and the diurnal change of NO and NO_2

WE have flown a radiometer from a balloon (a detailed description of which will be published elsewhere), which senses infrared emission from minor stratospheric constituents on the atmospheric limb. To provide a vertical profile of their concentration, the input mirror of the radiometer scans between 7° below and 2° above the horizontal, as illustrated in Fig. 1. The vertical field of view is 0.4° . Every 5.5 min the same mirror scans to an internal black body and to a space view at 18° upwards, thereby providing a two-point calibration.

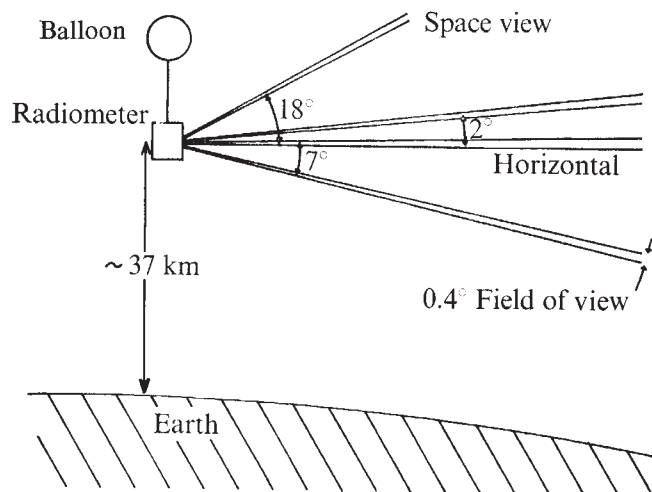


Fig. 1 Viewing geometry.

The optical system of the radiometer contains a rotating chopper which modulates incoming radiation at 195 Hz, and beam-splitters which divide the radiation between 3 channels with superimposed fields of view. Each channel contains an interference filter which selects the emission band of interest, a cell of the gas to be measured in which the gas pressure is modulated at a frequency near 20 Hz, and an infrared detector. The pressure modulator cell^{1,2} acts as a filter of very high resolution (down to $3 \times 10^{-3} \text{ cm}^{-1}$) which selects emission from the gas to be measured. The resulting increased sensitivity to this gas greatly reduces the relative sensitivity of the radiometer to changes in thermal emission from its input optics, and to emission from unwanted gases whose bands overlap the spectral region being observed. The detector receives components of radiation at both 195 Hz and 20 Hz, and by comparing the signals at each frequency we can almost completely compensate for the emission from unwanted gases. The device can be used even for corrosive gases, and has worked well, for instance, for NO .

The three channels measured emission from NO near $1,900 \text{ cm}^{-1}$, NO_2 near $1,600 \text{ cm}^{-1}$, and water vapour from 500 to 200 cm^{-1} . The 195 Hz water signal also measured the attitude of the radiometer by sensing the tropopause, above which the emissivity of the atmosphere drops from 1 to 0.7 within 3 km.

The sensitivity of the radiometer to each gas was determined in the laboratory by measuring the absorption of the gas in a cell in series with each pressure modulator cell. This sensitivity calibration is then independent of spectroscopic data, although such data were used to modify the water vapour calibration to compensate for different source and absorber temperatures between the laboratory and atmospheric measurements.

We present some results from two flights from the CNES launch site at Gap (44.2°N , 6°E) in southern France. The radiometer remained at its float altitude near 37 km for 5 and

7 h respectively, drifting almost due west towards 44°N , 0°E on each flight.

Figure 2 shows our measurements of the water-vapour mixing ratio. Between 15 and 30 km we find similar magnitudes to those of other workers^{3,4}, but above 25 km we find a substantial increase—reaching about a factor 2 by 35 km. Our systematic errors (due to uncertainties in altitude and attitude of the radiometer and in atmospheric temperature) give rise to significant absolute errors, but to rather smaller errors at one height relative to another. The increase in mixing ratio between 25 and 35 km is therefore at least a factor of 1.9—the results cannot be interpreted as a constant water mixing ratio. This increase supports the hypothesis that methane oxidation takes place in this region⁵, where the methane concentration decreases rapidly with increasing height^{6,7}.

Few measurements of water vapour above 30 km have been reported. Our results are different from those of de Jonckheere⁸, but are consistent with measurements by Scholtz *et al.*⁹ at 45 km. At higher latitude, Evans¹⁰ observed the mixing ratio to approximately double between 32 and 42 km, with a pronounced peak at 45 km.

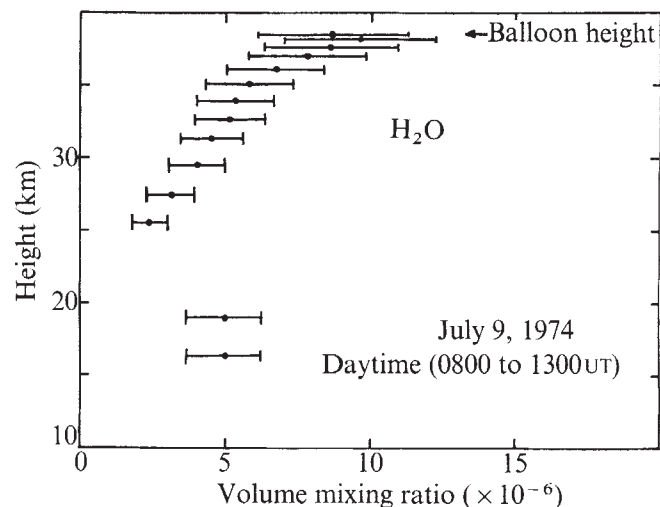
The later flight gave the first simultaneous measurements of changes in stratospheric NO and NO_2 from night to day. Our preliminary results are displayed in Fig. 3, and we find rather larger concentrations of these gases than other workers in the field. The probable errors at the present stage of analysis are less than a factor of 2, and again are mostly systematic errors in absolute concentrations. Changes in relative concentrations with altitude and time are much less uncertain.

The night-time concentration of NO was barely detectable relative to instrument noise, and was probably less than 0.8×10^{-9} by volume. The time of change and its sense (it rose to 15×10^{-9} by volume) agree with Patel's measurement at the single height of 28 km¹¹, although our daytime value is ~ 1.5 times larger, and over 5 times larger than daytime measurements by Schiff *et al.* up to 30.6 km (ref. 12).

Our NO_2 values are also somewhat larger than those obtained by solar absorption spectroscopy^{13,14}; they drop from 35 to 16×10^{-9} by volume at a height of 36 km. They are lower and have the opposite diurnal variation to those of Brewer¹⁵, but they are in broad agreement with those of Harries (personal communication).

Since the atmospheric path emitting to the radiometer is at least 300 km long, and the radiometer looks in a random direction relative to the Sun, the rapid rates of change of NO and NO_2 predicted at dawn by Whitten and Turco¹⁶ would be observed as time constants of ~ 15 min. We observed such a

Fig. 2 Water-vapour mixing ratio. The values < 20 km are from the 195-Hz signal, those > 25 km from the 20-Hz PMR signal. Random errors are equivalent to $\pm 0.1 \times 10^{-6}$ at 25 km and $\pm 0.4 \times 10^{-6}$ at 35 km. The data cannot be interpreted as a uniform mixing ratio.



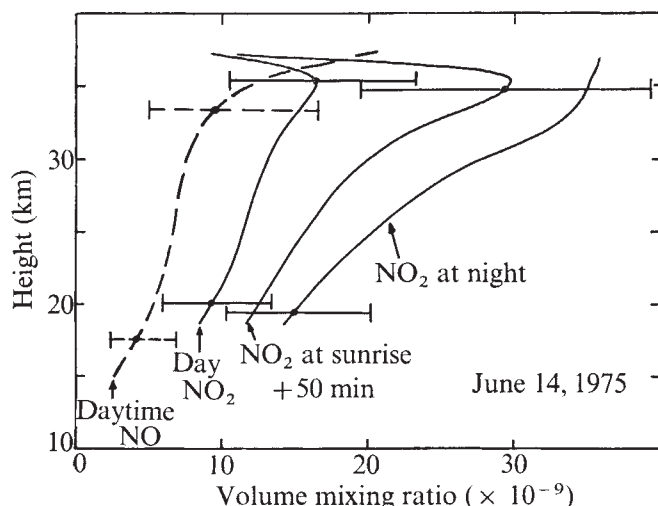


Fig. 3 NO and NO₂ mixing ratios. The errors are approximate, and contain both systematic and random components. Random errors are less than 0.5×10^{-9} for NO₂, and about 1.5×10^{-9} for NO. Systematic errors will be reduced by further analysis.

time constant for NO₂ at 36 km, but below 30 km the initial fall just after dawn was followed by a further slow fall over the next 2 h. Similarly, the lower altitude NO concentration continued rising for some hours after dawn. Within the errors of the analysis we have so far achieved, the sum of NO and NO₂ concentrations remains constant from night to day.

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Possible climatic impact of tropical deforestation

Of the various mechanisms suggested by which man might change the planetary climate, the removal of tropical rain forests to increase arable acreage seems to be one of the more imminent. For this reason we selected this as one of the first problems to be tested in our recently updated climate model. Bearing in mind the fallibility of computer simulations, we find overall global cooling and a reduction in precipitation: a larger tropical reduction being almost balanced by a subtropical increase.

The revised two-dimensional (zonal) atmospheric model (ZAM2)¹ uses basic conservation equations and parametrises the meridional transport by horizontal eddies using both model dependent and prescribed diffusion coefficients^{2,3}. Vertically, the atmosphere is divided into nine pressure levels, with three above the tropical tropopause. At each latitude of the 10° latitude grid, the lower boundary is considered to be subdivided into ocean, sea-ice, and land of differing types and elevations to properly represent the planetary surface. Although this does not give ZAM2 the spatial coherence of continents and oceans it does permit realistic treatment of boundary exchanges and energy and water balance. As the conditions at each latitude band change, the surface responds appropriately. Soil moisture is simulated by maintaining a surface water depth representing the running sum of precipitation, evaporation and runoff (a variable exponential loss rate designed to account for both runoff and percolation).

Roughly 34% of the equatorial zone from 5°N to 5°S is covered by tropical rain forest. To investigate the climatic effect of its removal, three simulation runs were made with ZAM2: first, a control run designed to represent present conditions with the rain forest albedo set at 0.07; second, the complete removal of the tropical rain forest simulated by increasing the albedo to 0.25, combined with an increased runoff rate and decreased evaporation rate; and third, 'wet' deforestation simulated by increasing the albedo to 0.25 only. Except for these modifications, all three simulations started with the same initial conditions and were run to equilibrium (which was determined by monitoring the rate of change of selected parameters, and required approximately six months integration). The constant sun version of ZAM2 was used, in which diurnal and seasonal variations are suppressed by imposing the annual mean solar flux appropriate to each latitude. This does not allow the strong north-south temperature gradients typical of winter, but has been shown to reproduce annual mean atmospheric conditions quite well⁴.

The chain of consequences following removal of the tropical rain forest as deduced from the ZAM2 results can be summarised as follows: deforestation → increased surface albedo → reduced surface absorption of solar energy → surface cooling → reduced evaporation and sensible heat flux from the surface → reduced convective activity and rainfall → reduced release of latent heat, weakened Hadley circulation, and cooling in the middle and upper tropical troposphere → increased tropical lapse rates → increased precipitation in the latitude bands 5 to 25°N and 5 to 25°S and a decrease in the Equator-pole temperature gradient → reduced meridional transport of heat and moisture out of equatorial regions → global cooling and a decrease in precipitation between 45 and 85°N and at 40 and 60°S. The changes were of essentially the same character in both perturbation cases, and seemed to be somewhat larger for the case of increased surface albedo only. We believe this is due to the greater cloudiness which occurs with the 'wet' deforestation. Contrary to expectation, most of the increase in cloudiness on this run occurred between 5 and 45°N.

Fig. 1 Predicted temperature changes due to removal of the tropical rain forest.

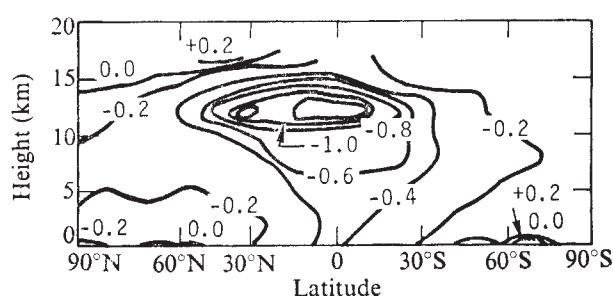


Table 1 Mean global water balance

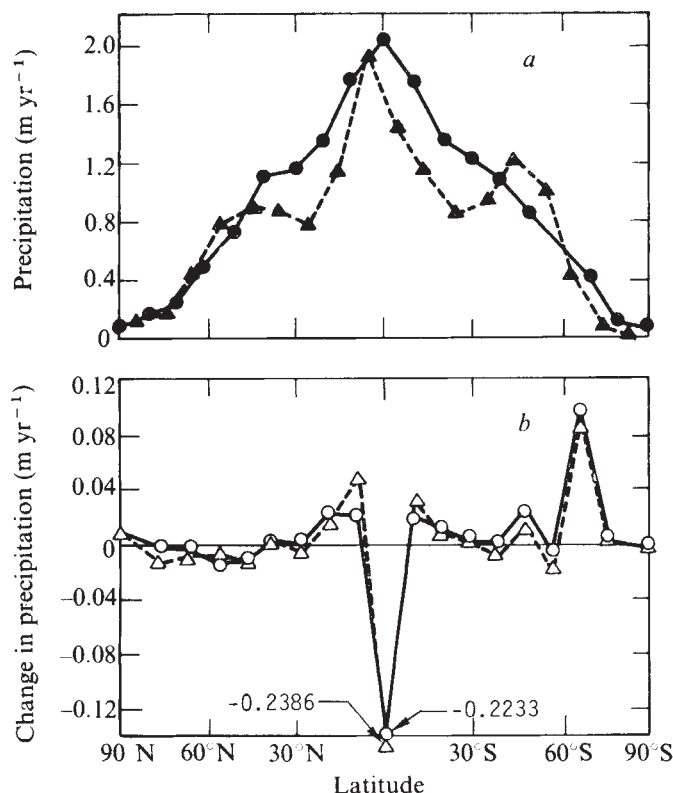
	Control	Complete deforestation	Albedo change only
Atmospheric water vapour content (gm cm^{-2})	2.4731	2.4032	2.394
Evaporation ($\text{gm cm}^{-2} \text{d}^{-1}$)	0.33323	0.32983	0.33016
Rainfall ($\text{gm cm}^{-2} \text{d}^{-1}$)	0.33305	0.32996	0.33169
Cloudiness	0.493	0.490	0.491
Atmospheric residence time (d)	7.4236	7.2847	7.2499

Figure 1 shows the latitude and height distribution of tropospheric temperature change for the complete deforestation simulation (perturbed minus control). The maximum temperature reduction below the tropical tropopause demonstrates the influence of reduced convective activity and subsequent reduced latent heat release. The poleward spread of this effect contributes to the decrease in static stability and increase in precipitation at 5 to 25°N and 5 to 25°S. Globally, the average surface temperature decreased 0.20 K for the completely deforested simulation and 0.30 K for the case of increased surface albedo only. The larger decrease in temperature in the latter case emphasises the importance of surface albedo and demonstrates the often overlooked tendency for self-compensation of geophysical processes in the real world. This represents the first time we have found a global temperature change not accompanied by greater surface changes at the poles than at the equator.

The zonal mean precipitation computed by ZAM2 during the control run is compared with observations in the upper part of Fig. 2. The lower part of the figure shows the changes in precipitation resulting from the two simulations of deforestation. The precipitation decrease in high northern latitudes reached 1.5 to 2.6% but amounted to only 0.9 and 0.7% when averaged over the globe for the two cases. The increases at 70°S are probably due to increased evaporation resulting from increased katabatic flow off Antarctica, but the reason for this increased flow has not yet been determined.

Table 1 summarises the global water budget as computed by

Fig. 2 Precipitation predicted by ZAM2. *a*, Comparison of control case (●) with observations compiled by Sellers⁵ (▲). *b*, Change in precipitation (perturbed minus control) for complete deforestation (○) and albedo change only (△).



the three model runs. These show what has now come to be a familiar pattern; global cooling leads to reduced atmospheric water vapour content (reinforcing the cooling by a reduced 'greenhouse' effect). This in turn leads to a reduction in global mean precipitation, which is consistently smaller than the reduction in precipitable water because of an acceleration of the atmospheric water cycle (revealed by the decrease in residence time of water vapour in the atmosphere). These effects are in turn accompanied by an even greater cooling in the upper tropical troposphere, a steepening of lower tropospheric lapse rates, a lowering of the tropical tropopause and a warming of the lower tropical stratosphere (see Fig. 1).

In this type of experiment the results should be viewed primarily as indications of possible change and not as absolute indicators. Although as yet, no claim can be made that this cascade of events would occur in the real atmosphere, it is consistent with the prevailing conception that lapse rates in the lower troposphere and the height of the tropical tropopause are determined largely by moist convection. Also, in a parallel study, the model duplicated quite well the published results⁶ of a three-dimensional general circulation model test of the climatic impact of increased albedo by denudation of the Sahara.

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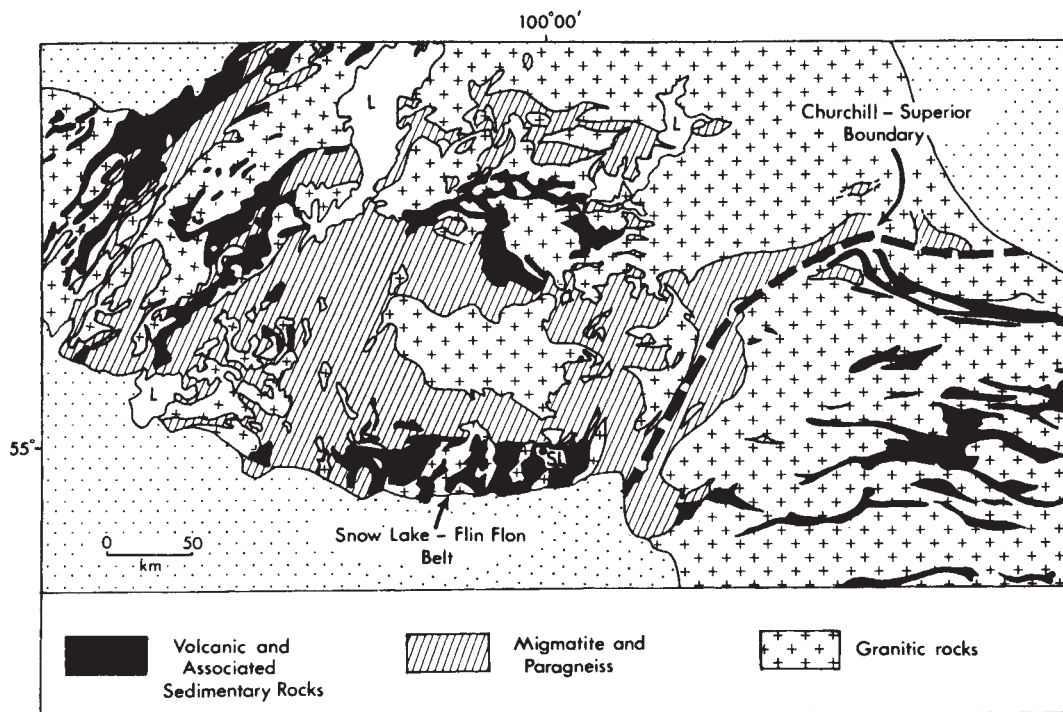
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Evidence for a Proterozoic greenstone belt from Snow Lake, Manitoba

THE Archaean-Proterozoic boundary, it has been suggested, marks a fundamental change in the behaviour of the Earth, ending an early phase of cratonisation and the beginning of a period characterised by stable shelf sedimentation and linear orogens^{1,2,3}. Almost all Archaean terrains, other than those dominated by granulites and migmatites, seem to be characterised by a variety of granitoid rocks intimately associated with ultramafic through mafic to calc-alkalic sequences, and their sedimentary associations. These greenstone belt assemblages are remarkably similar from one craton to another; they are usually folded into keel-shaped troughs, subjected to greenschist facies metamorphism and usually show similar structural styles which suggest that they were acted on by predominantly vertical forces. Their

Fig. 1 Regional setting of the Flin Flon–Snow Lake belt at the southern margin of the Churchill Province. The Superior Province lies to the east. L, lakes; S.L., Snow Lake.



alleged antiquity (2,000 Myr or more) and widespread distribution have been attributed to either a change in the geothermal gradient as the Earth became colder³ or the existence of a relatively thin, Archaean, continental crust^{4,5}.

Detailed proposals for the origin of greenstone belts are more varied and include models that compare the volcanic–sedimentary sequences with: (1) primitive crustal remnants⁶, (2) volcanism associated with continental rifting¹ and (3) island arc sequences⁷. The last of these is particularly interesting because it implies that plate tectonic processes were common in the Archaean and, as a corollary, that greenstone belts or their equivalents were formed throughout geological time. This note presents some new Rb–Sr data which indicate that not all greenstone belts are restricted to the Archaean. If this conclusion is accepted then the uniqueness of Archaean magmatic processes diminishes, and we are faced with attributing the origin of greenstone belts to mechanisms similar to those we find operating at the present time.

The Flin Flon–Snow Lake volcanic–sedimentary belt at the southern margin of the Churchill Province in northern central Manitoba and Saskatchewan (Fig. 1)^{8,9} has traditionally been regarded as Archaean on the basis of its approximate east–west trend and its similarity to Archaean greenstone belts in the nearby Superior Province to the east¹⁰.

Numerous high grade base-metal deposits related to the volcanic successions make this one of Canada's major metal-producing areas. Deformation, metamorphism and plutonism have been ascribed, in large part, to the Hudsonian Orogeny¹¹. Age determinations have indicated some evidence for an Archaean history for the Hanson Lake area, near the western part of the belt¹², but of an Aphebian age (1,700–1,800 Myr) of magmatism for the Flin Flon–Snow Lake area (refs 13, 14 and G. R. Josse, unpublished).

Although not continuous with the type succession, the major rock units at Snow Lake are correlative with the Amisk metavolcanics and overlying Missi metasediments at Flin Flon^{15,16}. Full details of the Amisk and Missi Groups at Snow Lake are given in Table 1. The entire succession has been deformed into three major fold sets and metamorphosed with grade increasing from the biotite zone in the south to the sillimanite–almandine zone in the gneiss dome terrain to the north (see Fig. 2). Major stratigraphical units can be traced across the isograds. The cores of the gneiss domes are granitoid rocks with chemical compositions similar to the more felsic of the volcanics.

Table 2 summarises the whole-rock Rb–Sr isochron results from six suites collected from the Snow Lake area. The localities are shown in Fig. 2. Of the six isochron ages listed in Table 2, none is Archaean, none is older than

Table 1 Stratigraphy of Snow Lake

Missi Group	Arkosic siltstone and sandstone, typically cross-bedded; minor conglomerate, greywacke	Post-Missi (?) intrusions: gabbro, diorite, granodiorite, granite
	— Minor Unconformity —	
Amisk Group	Pelite and greywacke turbidites	Plutonic and hypabyssal equivalents (?): 'Quartz-eye' dacite
	Interlayered tholeiitic and calc-alkalic volcanics; basalt, andesite, and dacite breccias, tuffs and flows; calcareous and pyritic tuffs; related intrusions	Granitoid gneiss domes of Herblet and Squall Lakes
	Pillowed and massive basalt flows; related intrusions	

Table 2 Analytical results

Locality	Petrographic notes	No. of points	Initial ratio	Age (Myr)	MSWD
A Amisk Group—due south of Snow Lake	Amphibolite, biotite-hornblende gneiss, garnet-biotite gneiss and schist derived from pillowed tholeiite, basalt breccia, andesite, and fine-grained andesite and dacite pyroclastics	5	0.7020 ± 0.0005	$1,720 \pm 45$	2.3
B Amisk Group—due north of Snow Lake	As above	4	0.7017 ± 0.0007	$1,720 \pm 40$	3.4
C Triangle Dome	Very uniform pink, granoblastic, medium-grained biotite-hornblende granites with approximately equal amounts of microcline, sodic plagioclase and quartz	4	0.7017 ± 0.0010	$1,750 \pm 70$	2.8
D East Dome	As above	6	0.7024 ± 0.0018	$1,720 \pm 110$	5.3
E Squall Dome	Recrystallised uniform leucogranodiorites, one with minor garnet; schistose and gneissic variants with up to 10 percentage biotite plus muscovite, also lens of hornblende-biotite metadiorite	4	0.7014 ± 0.0009	$1,690 \pm 60$	3.1
F 'Quartz-Eye' Dacite	Biotite-quartz-plagioclase schist, with variable small amounts of garnet and hornblende. Polycrystalline quartz 'eyes' derived from phenocrysts in fine granular matrix. Contains inclusions of enclosing metavolcanics	5	0.7019 ± 0.0006	$1,510 \pm 110$	3.6

All errors given at the 2σ level. All ages calculated using $\lambda = 1.47 \times 10^{-11} \text{ yr}^{-1}$.

1,750 Myr and all reflect a Proterozoic event which we believe corresponds to the age of magmatic activity. Other than that of the 'quartz-eye' dacite, the isochron ages fall within a fairly restricted range of between 1,750 and 1,690 Myr. We have also listed in Table 1 a statistical parameter, the mean square of the weighted deviates (MSWDs)¹⁷, which gives some idea of how well the points fit a straight line. For our isochrons the MSWDs indicate that the points fit the line within experimental error.

It is possible to interpret the analytical data in two different ways, one of which we have mentioned. The other interpretation assumes that the rocks are considerably older and that the Proterozoic ages are the result of a resetting

and updating of the Rb-Sr systems by the Hudsonian Orogeny. This we feel unlikely on the following grounds: First there is very little scatter of the data other than what can be attributed to analytical uncertainty. The low MSWDs (between 2 and 5) suggest that there is little 'geological noise' of the type to be expected from a disturbance of the Rb-Sr systems. Second, all of the rocks have relatively low, uniform initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios which suggest that these rocks have not had an extensive earlier history. Third, single-stage model-lead ages for the stratabound sulphide deposits, which on geological grounds are coeval with their host rocks, predominantly volcanics of the Amisk Group, fall between 1,900 and 1,780 Myr (ref. 14). Although

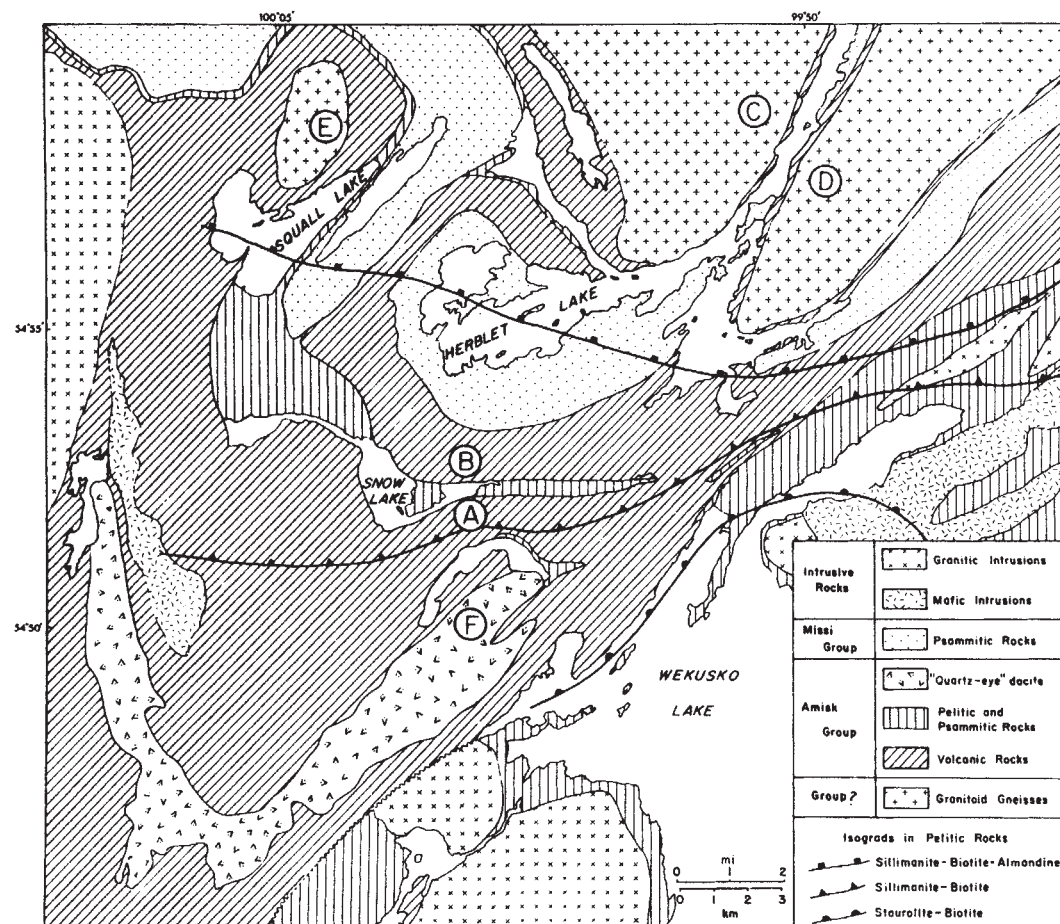


Fig. 2 Detailed map after Froese and Moore¹⁶ showing sample localities A-F. For locality descriptions see Table 2.

these ages are strongly model-dependent the isotopic data can be interpreted to be consistent with our Rb-Sr results. Finally, if the Hudsonian Orogeny were responsible for the complete resetting of the Rb-Sr systems then a process would have to be invoked which behaved independently of chemical composition, grain size and metamorphic grade.

We prefer the simplest interpretation of the isotopic data, which considers the volcanics and gneiss domes as magmatic equivalents erupted just before the onset of the Hudsonian Orogeny. It is significant that there is no evidence either in our data or those of other laboratories for an Archaean crustal history. In spite of the similarities of the rocks at Snow Lake to Archaean greenstone belts, the radiometric data consistently indicate Alpehian volcanism. The close association of stratabound sulphide deposits to Proterozoic, rather than Archaean, volcanicity has some profound implications, particularly in the context of base-metal exploration in the Canadian Shield.

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Evidence for pre-Jurassic subduction in western Antarctica

THE South Shetland Islands (from King George Island to Livingston Island) are situated on a small crustal plate bounded by incipient back-arc spreading along the axis of Bransfield Strait to the east, a well defined oceanic trench to the west (along which subduction has apparently now ceased) and transverse faulting to the north and south¹ (Fig. 1). The discovery of glaucophane-schists on Smith Island (Fig. 1, inset) suggests that subduction may have begun as early as the Upper Palaeozoic.

The geological history of the South Shetland Islands (which includes the Elephant and Clarence Islands group) is dominated by continuing volcanism from the (?) Jurassic to the present². A structurally deformed sedimentary sequence of presumed Upper Palaeozoic age crops out on Livingston Island^{3,4}. Structurally deformed phyllites, schists and rare marbles, constituting the Elephant and Clarence Islands group, have been assigned a (?) Precambrian

age^{4,5} but Smith Island is known only to consist of "base-metamorphic rocks" (see ref. 6).

Cape Smith on Smith Island, and Barlow Island are formed of grey-green strongly schistose rocks exhibiting fine colour banding (mainly shades of green but including dark, grey-blue horizons) and small-scale structural features; the banding is dominantly parallel to the schistosity but marked local discordances occur. A buff coloured, fine grained, clastic dyke, indurated and pinched out laterally, cuts the schistosity at Cape Smith, and quartz veining, both concordant and discordant, also occurs.

Thin-section analysis of specimens collected from the localities has revealed five metamorphic rock types: epidote-quartz-garnet-glaucophane-semischist; epidote-albite-chlorite-glaucophane-schist; epidote-chlorite-glaucophane-schist; epidote-glaucophane-schist; and an epidote-albite-chlorite rock.

The mineralogy of these rocks is relatively simple. Yellow, slightly pleochroic epidote (pistacite) forms layers of tiny granules and stubby prisms, and idiomorphic acicular glaucophane (α =colourless; β =deep blue; γ =colourless) is aligned parallel to the schistosity. Mosaic quartz and albite are the commonest associates and probable metamorphic differentiation of these four phases has created layers and lenses virtually exclusive of other minerals. Prochlorite (?) is also present and minute granules of accessory sphene (?) form discontinuous, sinuous trains. Xenomorphic pale yellow garnet (composition undetermined) occurs in one specimen, often altered to a red-brown mineral and intimately associated with epidote, both externally and internally. This specimen also contains radiating sheaves of stilpnomelane largely restricted to the epidote-rich layers intercalated between laminations of finely crystalline quartz (possibly originally chert). With that exception, all of the rocks have a basic composition⁷ and probably represent original basic igneous rocks.

The most conspicuous structural feature is a regular schistosity, the planes of which exhibit a mineral lineation. Both of these features have subsequently been deformed, resulting in tight asymmetric folds of several centimetres wavelength. Kink bands of 1 or 2 mm diameter are irregularly developed. Stereographic projection of limited structural data also indicates two phases of deformation.

Although these rocks are the first glaucophane-schists to be recorded *in situ* from western Antarctica, two glaucophane-schists were dredged from south of Clarence Island⁸, and five from the northern Scotia Ridge by RRS Shackleton in 1972 (Fig. 1).

No inference can yet be made regarding the provenance of the northern Scotia Ridge specimens. The ubiquitous association of ultrabasic (mostly serpentinitic) rocks with glaucophane-schists may be represented here by the serpentinite-dunite of Gibbs Island and the glaucophane-schists of Smith Island, which suggests that the specimens described by Tyrrell originated within the South Shetland Islands. Texturally, Tyrrell's specimens are distinct from those of Smith Island.

Geophysical evidence suggests that a metamorphic basement stretches from the Elephant and Clarence Islands group as far south as Livingston Island⁶; this probably extends to include Smith Island.

Apart from textural differences, all of the glaucophane-bearing rocks from the South Shetland Islands have similar mineral assemblages which are atypical of both greenschist and glaucophane-schist facies. In particular, the coexistence of epidote, garnet and soda-amphibole is typical of conditions transitional between these two facies⁹.

Lithological and structural similarities^{10,11} between the Elephant and Clarence Islands group and the South Orkney Islands are supported by geophysical evidence¹². The latter islands largely comprise metamorphic rocks

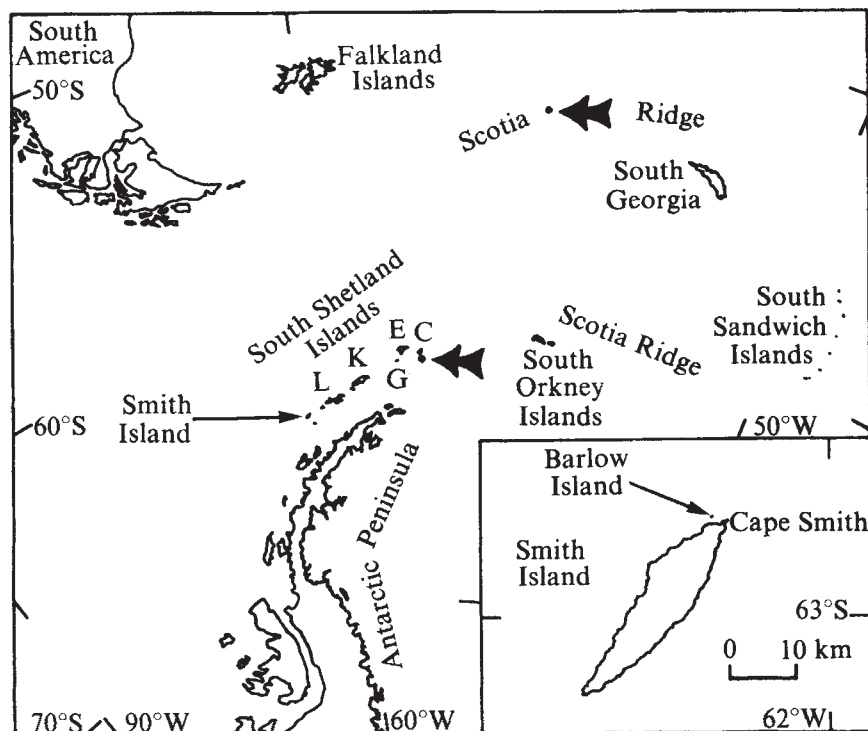


Fig. 1 Map of the Scotia Ridge showing the occurrences of glaucophane-bearing rocks. Arrowed solid circles, dredge sites; C, Clarence Island; E, Elephant Island; G, Gibbs Island; K, King George Island; L, Livingston Island. Inset, Smith Island.

belonging to the highest grade of the greenschist facies' but with a local development of quartz-albite-sillimanite-schists at Mansfield Point on Coronation Island (South Orkney Islands) which may possibly be attributable to metasomatism from an unknown source at depth.

Whereas lithological differences between the deformed metamorphic rocks of the Antarctic Peninsula and those of the offlying islands preclude correlation^{4,13}, the contrasting physical conditions of metamorphism have not been emphasised previously. The rocks of the Antarctic Peninsula, metamorphosed under high-temperature, low-pressure conditions, have been specifically compared with the Ryoke-Abukuma Metamorphic Belt of Japan¹⁴. Conversely, those of the South Shetland and South Orkney Islands, formed in low-temperature, high-pressure conditions, are more similar to those of the Sanbagawa Metamorphic Belt of Japan^{15,16}. It is therefore proposed that a paired metamorphic belt, analogous to the Ryoke-Abukuma/Sanbagawa paired belt, exists in western Antarctica, and its formation is ascribed to the subduction of oceanic crust along a Benioff Zone beneath either an island arc or a continental margin¹⁷. The relative positions of the inner and outer metamorphic belts indicate subduction from west to east.

There is no evidence to indicate conclusively the age of the initial metamorphism of the rocks of the metamorphic complex. A pre-Jurassic age is, however, indicated by the unmetamorphosed and largely undeformed Jurassic igneous intrusions of the Antarctic Peninsula¹³. Radiometric ages¹⁸ suggest that a significant metamorphic and deformational event occurred in the Upper Palaeozoic but this may be considered a minimum age. Although there is no radiometric evidence to suggest a metamorphism before the Upper Palaeozoic, it cannot be shown that the formation of the two metamorphic belts actually occurred at this time, but they must have existed to account for the similar radiometric ages from the Antarctic Peninsula and the South Orkney Islands. Accepting the reconstruction that there was a continuous Andean-western Antarctic cordillera at least until the late Mesozoic¹³, it is interesting that a crossite-bearing, paired metamorphic belt present in central Chile has been assigned¹⁹ an Upper Palaeozoic age.

The major fault¹⁷ or crustal suture²⁰ which seems to characterise paired metamorphic belts can only be inferred at present, but it may be expected on the South Orkney Islands platform (perhaps located along the 2.5 km deep, buried trench to the south of the islands²¹) and in Bransfield Strait, where such a feature may have had a major influence on the evolution of the back-arc spreading.

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Ceramic steel?

ZIRCONIUM DIOXIDE (zirconia) has three allotropes: monoclinic, tetragonal and cubic. The transition between the first two involves a large volume expansion which prevents the refractory properties of pure zirconia being used in structural ceramics. The disruptive phase transformation can be suppressed by total stabilisation in the cubic form, but it is generally recognised that the most useful mechanical properties are obtained in multiphase material known as partially-stabilised zirconia (PSZ). Garvie and Nicholson¹ have demonstrated that a fine-scale precipitate of monoclinic zirconia in a cubic stabilised matrix enhances the strength of PSZ. Here we report that a dispersion of metastable tetragonal zirconia in cubic zirconia

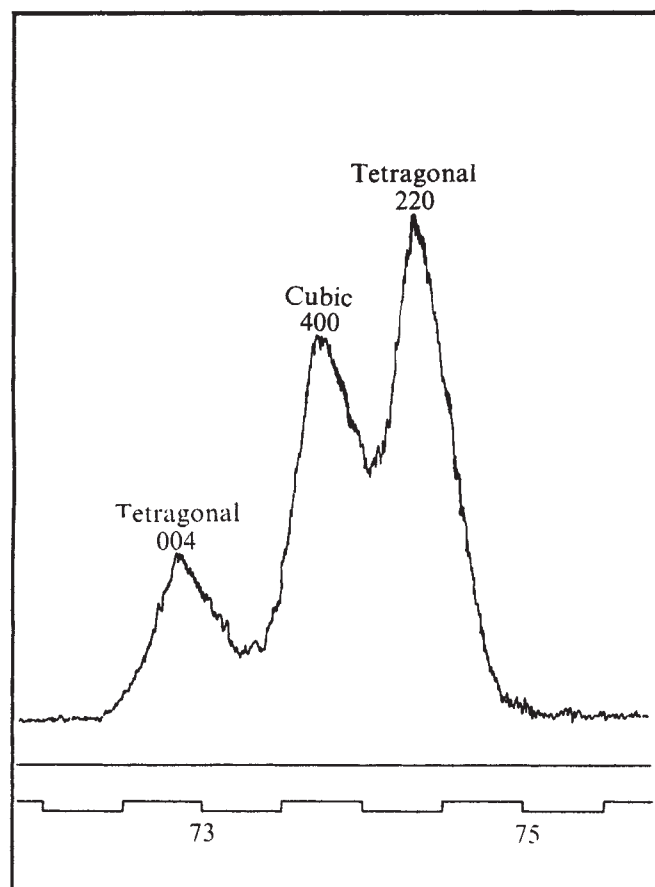


Fig. 1 X-ray diffractometer trace of the {400} peaks showing cubic (400) and tetragonal (004) and (220) reflections. Trace from an as-fired surface of zirconia partially-stabilised with lime ($\text{CuK}\alpha$ radiation).

can also be achieved, and that this gives rise to another, more powerful, strengthening mechanism.

The transformation between tetragonal and monoclinic zirconia is believed to be martensitic²; it is fast and diffusionless. It is claimed that the tetragonal phase cannot be

retained in bulk material to room temperature and atmospheric pressure by any quenching procedure^{2,3}. Metastable tetragonal zirconia is known to exist at room temperature only in particles of less than ~ 30 nm diameter; in this case stability is due to the lower surface energy of the tetragonal phase compared with monoclinic⁴ (although this explanation is not universally accepted⁵).

Our evidence for the existence of tetragonal zirconia in sintered bodies of lime-stabilised zirconia is extensive. We suspect that the magnesia PSZ material that has been reported to have high strengths⁶ would show the same structural features. X-ray diffraction patterns have been obtained from foils thinned by an ion beam and un-machined surfaces, and may be clearly indexed as a mixture of cubic and tetragonal phases (Fig. 1). Transmission electron microscopy of these thinned samples reveals a duplex structure in which second phase particles up to ~ 100 nm in diameter are unambiguously tetragonal (Fig. 2). Carefully prepared samples show no evidence of the monoclinic phase unless the diameter of the second phase is > 100 nm. This conclusion is supported by thermal expansion measurements which exhibit no discontinuity on heating to $1,500^\circ\text{C}$; a sudden change in length is normally a sensitive indicator of the presence of monoclinic zirconia (Fig. 3).

The tetragonal phase is metastable and can be transformed readily to monoclinic, for example by diamond grinding the surface or by pulverising during preparation for X-ray powder photographs. The stabilisation of the tetragonal phase is thought to arise from a combination of the surface energy effect discussed above and the constraint of the rigid matrix which opposes the transformation to the less dense monoclinic form⁷. As a result the transformation can occur locally once surface constraints are removed; it is probably triggered by shear strains. The transformation has been observed in thin foils during exposure to the electron beam in an electron microscope.

The presence of a tetragonal phase that can revert to the stable monoclinic form has a number of important consequences. The first is a significant increase in strength. Mean transverse rupture strengths of 650 MPa have been measured in lime-stabilised zirconia deformed in four-point bending. This strength is comparable with the highest values reported for oxide ceramics—in fine grained alumina⁸. By contrast the strength of PSZ containing a purely monoclinic phase is 250 MPa. The increase in strength is achieved by an increase in the work of fracture, up to 500 J m^{-2} , rather than by a reduction of defect size (usually grain size or porosity) as in many other strong ceramics. Part of the increased work of fracture probably arises from a dispersed phase interacting with the crack front, as discussed by Bansal and Heuer⁹. A more important contribution comes from the absorption of energy during the martensitic transformation of tetragonal particles to monoclinic (as in the TRIP steels¹⁰).

A further contribution to strengthening arises from surface finish. By contrast with other brittle materials, grinding the surface increases, rather than reduces, the strength compared with a polished finish. This difference is associated with an increase in the monoclinic content at the surface on grinding and is illustrated in Table 1. The proportions of the phases recorded there were estimated

Table 1 Influence of surface condition on strength and phase content (determined by X-ray diffraction) of zirconia partially stabilised with lime

	Transverse rupture stress ($\pm$ s. d.) (MPa)	FWHM (111 cubic peak) (2θ)	% monoclinic at surface ({111} peaks)	% tetragonal at surface ({400} peaks)
As-fired	Not determined	0.22°	0	53
Diamond ground	600 ± 11	0.46°	17	36
Ground and polished	490 ± 30	0.35°	9–12	41
Ground and annealed for 1 h at 850°C	500	0.36°	16	37

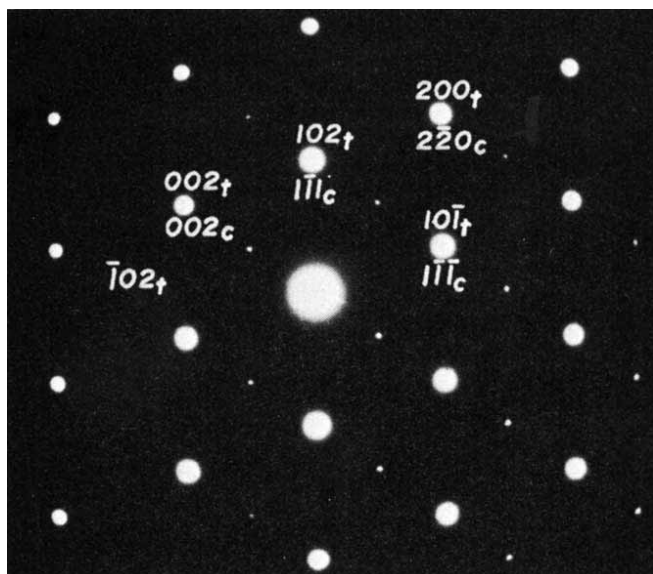
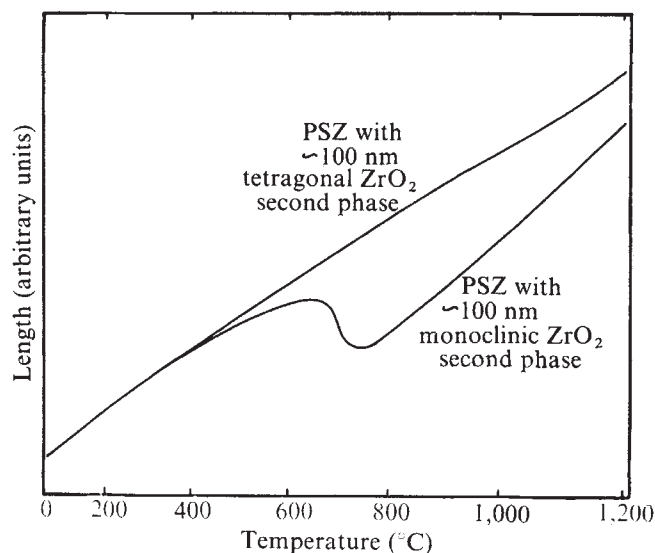


Fig. 2 Electron diffraction pattern of [110] cubic pole also showing tetragonal spots of [010]. Forbidden tetragonal spots occur due to double diffraction.

from the relative areas under the $(11\bar{1})_m$, $(111)_m$ and $(111)_c$ and the $(004)_t$, $(220)_t$ and $(400)_c$ (referred to as $\{400\}$) X-ray diffraction peaks¹¹. An as-fired surface is free of the monoclinic phase, but this is generated (by transformation of the tetragonal phase) on grinding; this process induces compressive strains at the surface and increases the transverse rupture stress. Careful polishing removes some of the monoclinic material, without transforming any further tetragonal, and so reduces the surface strain and breaking stress. A similar reduction of strength can be achieved by annealing which removes the strain (indicated by FWHM) without reducing the surface content of monoclinic phase. Increases in the monoclinic content at the surface are matched by decreases in tetragonal content.

It may be noted that alloy systems based on zirconia have many features in common with systems based on iron, including three allotropes, martensitic transformations and metastable phases. The vast range of materials

Fig. 3 Dilatometer traces obtained from PSZ with tetragonal and monoclinic second phases.



with very different properties formed in the iron system lead us to believe that our strong and comparatively tough alloys are but first developments in zirconia-based ceramics. We believe that, in development potential, PSZ can be considered a ceramic analogue of steel. Patents covering materials and processes have been applied for.

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Pulsating turbidity currents with relationship to high swell and high tides

THE Rio Balsas of Mexico has built a delta into the Pacific at latitude 18°N, virtually covering the narrow shelf, where the seaward slope is cut by numerous valleys. In 1967, during a period of large swell, Reimnitz¹ made a SCUBA dive to 18 m in a tributary of the Rio Balsas Canyon (Fig. 1) and encountered downcanyon current pulses estimated at more than 1 m s⁻¹. We visited the area on the RV Ellen B. Scripps in April 1975 and placed a current meter in Rio Balsas Canyon at a depth of 285 m. During the 5-d recording, a period of rather heavy swell occurred. This was indicated on our tide record (taken ~0.5 km inside the river estuary) by wave-induced oscillations with amplitudes up to 20 cm at periods of about 3 or 4 min. These persisted for approximately 36 h (Fig. 2). High swell was also observed on our fathometer records during traverses nearshore, and wave heights up to ~5 m were seen, considerably larger than during the rest of our study.

The computerised current meter record showing the times and velocities of upcanyon and downcanyon flows clearly indicates a change in the pattern during the period of high swell. Before and after the large waves, there was an alternation of flows up and down the canyon with velocities not exceeding 35 cm s⁻¹. The period of high swell, however, includes a downcanyon surge of 55 cm s⁻¹, followed by low currents lasting for 16 h, and then by six downcanyon surges all exceeding 50 cm s⁻¹ (with two attaining 62 cm s⁻¹). These strong surges were each separated by intervals of ~1 h, during which there was no appreciable current, and seemed to be a type of low speed turbidity current. This contention is supported by our finding a coating of fine sand and silt on the case of the current meter that was positioned 3 m above the bottom.

A possible relationship of the surges to two high spring tides at that period should be considered seriously, in spite of the fact that the six closely spaced surges occurred somewhat before high tide. Tides in a narrow estuary or river

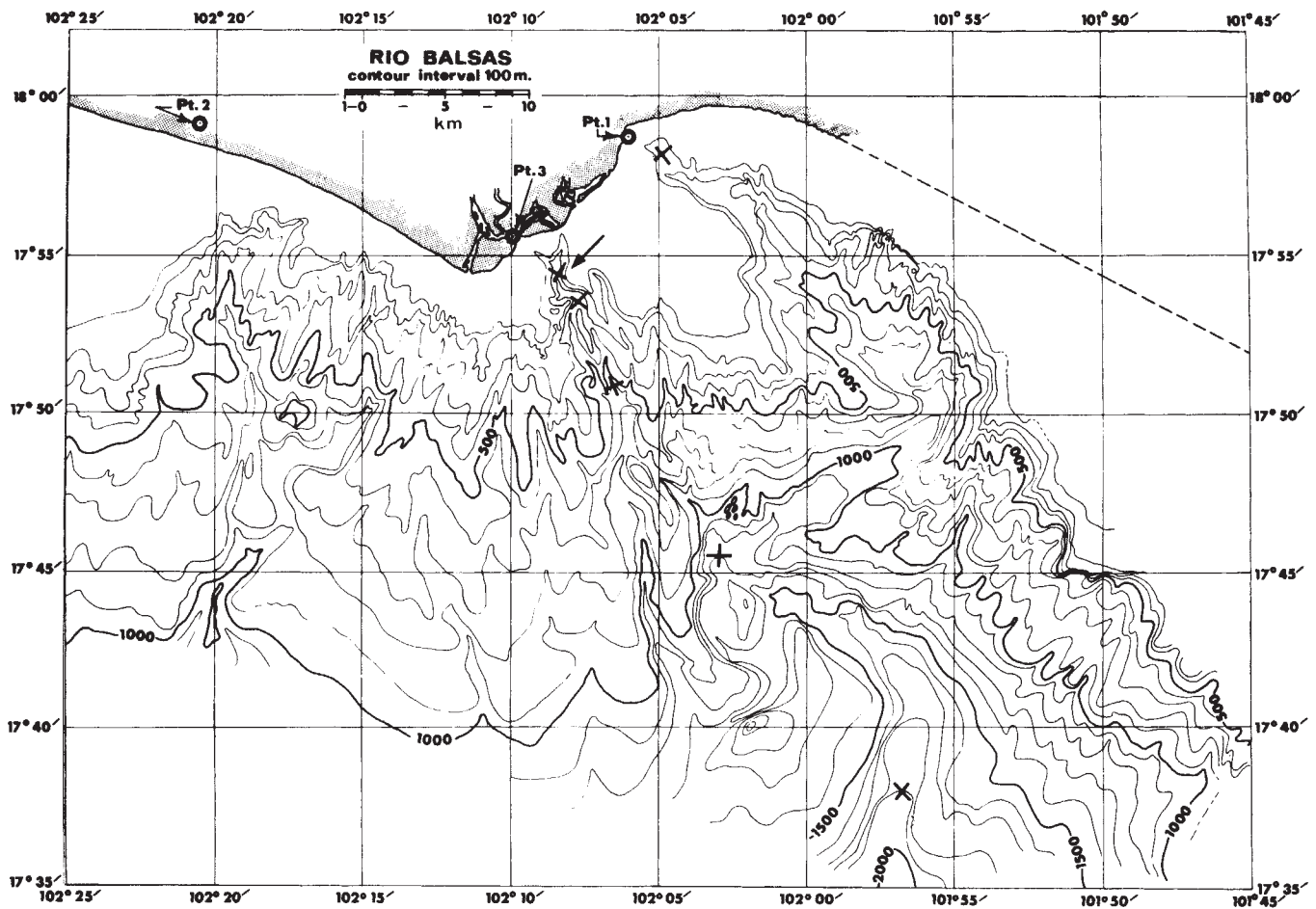


Fig. 1 The submarine valleys off the Rio Balsas Delta showing the location where the presumed turbidity current pulses were measured by a current meter (see arrow). Other crosses give locations of other current meters emplaced during the Rio Balsas Expedition.

mouth are always later than those on the open coast. Tide tables show many instances when the time difference is a matter of hours. If we assume that a difference of about 2 h existed here, the six surges could have occurred within the bounds of the second high tide. That this was actually the case is suggested by the fact that the time interval between the first large surge and centre of the six surges is very close to the interval between the two high tides.

Assuming that this relationship existed, it is possible to offer an explanation for the turbidity currents. Inman *et al.*² have found that storms with strong onshore winds cause water to set up along the shore, but with less rise at the head of a submarine canyon than over the adjacent shelf on either side. This imbalance develops a strong flow towards the head of the canyon and a return flow along the canyon. The same group also postulate that edge waves along the coast cause long period current oscillations that suspend sediment in the canyon heads, which, in storm conditions, may be capable of producing the density conditions necessary for turbidity currents with pulsating flows. On several occasions, we have had current meters carried down the canyons during these storm conditions at La Jolla, California³. Genesseeux⁴ also observed pulsating down-canyon flows off the Var River in Southern France during flood and storm conditions. Pulsating turbidity currents are, however, not necessarily due to storm conditions. Lambert and others⁵ obtained a record of turbidity currents with pulsating flows of 30 cm s^{-1} in the Wallensee (in Switzerland) during heavy runoff from melting snows in the neighbouring mountains. Also, the pulsating flows observed

by Reimnitz, mentioned previously, were during high swell rather than storm conditions.

Thus, it seems likely that large swell alone without high winds can produce a set-up of water along the shore and the necessary flow toward the canyon heads to produce pulsating turbidity currents. At the Rio Balsas Delta, it seems probable that the first strong downcanyon surge was developed only after the high tide had combined with the flows along the shore caused by the high swell. Then the rapidly falling tide prevented a recurrence until a considerable set-up of the water from the growing swell conditions was again favoured by the second rising tide, and the surges were able to repeat with only an hourly interval necessary for building up a head. After the sixth surge, the swell began to decrease and the falling tide helped bring the event to an end. It is significant that our current meter records, which were continued at somewhat greater depths in the same canyon head during the next week, showed no repeat of the strong surges, nor was there a repeat of either the large swell or of the high spring tides.

Although there is much evidence that turbidity currents can extend seaward for many miles, that may be true only during major high-velocity currents, like those of the Grand Banks earthquake^{6,7}. There is evidence that the pulsating currents in the canyon off Rio Balsas did not extend any great distance. We had two other current meters in operation in the same canyon at distances of 17 and 36 km seaward of the point where the pulses were observed. Neither of these records showed any indication of unusual downcanyon flow during or after the period of large swell.

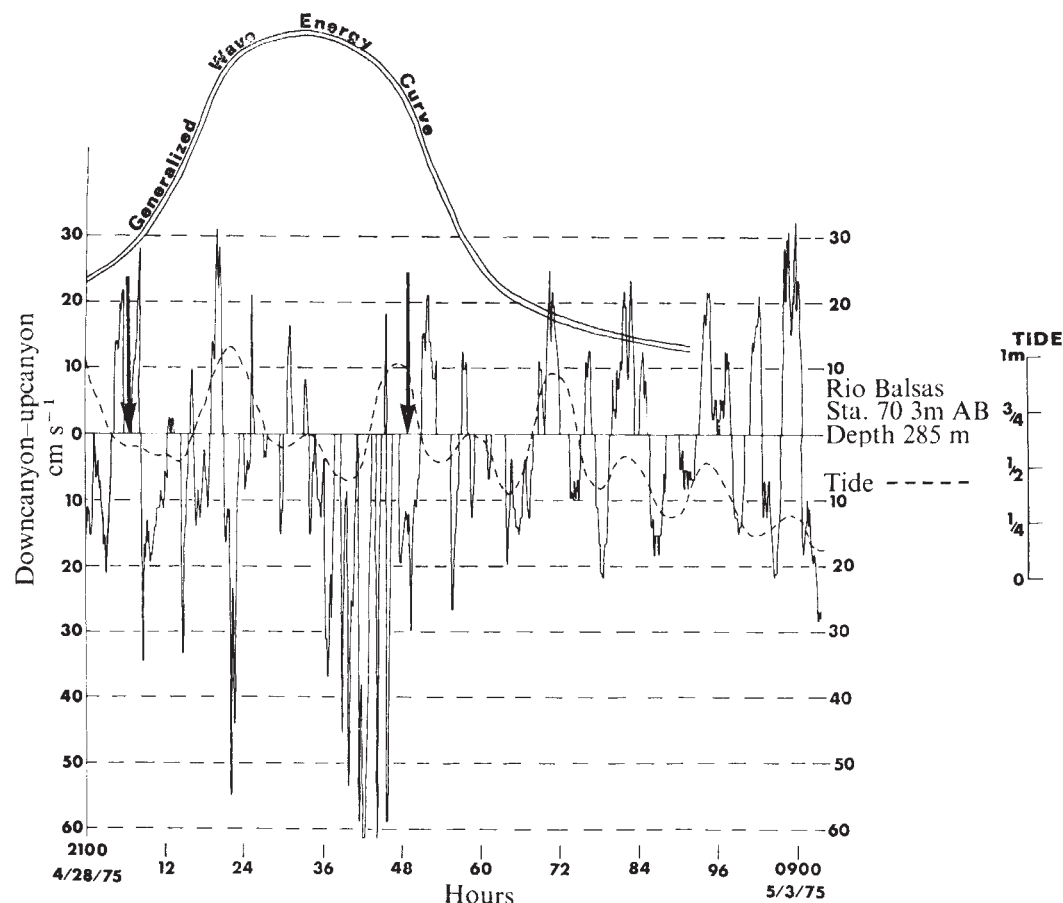


Fig. 2 The 108-h record of up- and downcanyon flows in the head of Rio Balsas Canyon at a depth of 285 m. Note the confinement of the large downcanyon surges to the period of large swell shown by the wave-energy curve. The two vertical arrows show the limits of large amplitude, short-period oscillations on the tide gauge inside the river mouth. The time of high tide at the gauge was probably appreciably later than at the canyon head.

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Hermatypic coral growth banding as environmental recorder

STUDY of incremental banding in coral skeletons was initiated by Ma¹ and later extended by Wells² and others³. More recently, discrete annual banding in the skeletons of certain hermatypic corals^{4,5,6} has been described. Here we present an analysis of annual band width measurements from Bermuda corals which relates, through regression techniques, coral band time series to air temperature and air pressure variations. Our results indicate that coral bands record important aspects of their environment and

therefore become useful where reconstruction of palaeoclimatic variables is of interest. Specifically the coral time series may be used as a palaeotemperature indicator or, when coupled with relatively well established palaeotemperatures, for palaeobarometric pressure determinations. Derived series of otherwise unobtainable palaeovariations are important not only for work on coral physiology, but also for construction and testing of climatic models; in this later instance information on palaeopressure is particularly desirable.

We chose Bermuda as a study area for a variety of reasons. Previous work³ had revealed annual banding in the skeletons of the genus *Diploria*, correlations among individual coral heads, and low growth rates which allowed a large number of years to be recorded within a small coral. Geographically, Bermuda is at the northern limit of hermatypic coral distribution, providing an ideal site for the study of limiting factors of growth and for the monitoring of long term climatic changes. Because of Bermuda's near mid-oceanic position, the reconstruction of any climate variables at this site is of intrinsic meteorological interest.

Between May and June, 1973, 40 specimens of the brain coral *Diploria* species, ranging from 10 to 40 cm in height were collected in 2–5 m of water at various locations off the south and east shore of Bermuda (Spanish rock to the east of St Catherine's point). Specimen preparation and method of band measurement are described in Dodge and Thomson³. Briefly, coral sections were obtained and X radiographed to reveal annual bands. Six specimens were rejected because of small size (< 23 measurable bands), two because of non-hemispherical shape and heavy erosion by borers, two because of unclear annual bands and three because of faulty sectioning. Band widths for the 27

retained samples were measured and summarised into whole coral series in index form⁷, which were then combined into a master chronology. Since banding patterns between individual heads were similar, summarising all corals into a master series maximised common variability yet minimised random and individual variations. Although measurable bands extended to the year 1900 AD in some corals, the number of individuals included in the chronology drop out at older times. To maintain the sampling standard deviation within a factor of 2 of the best sampled years, the series used in the regression analysis was terminated in 1905.

The choice of physical variables for comparison with the growth series was a compromise between availability of long records and known relationship to variables affecting corals (including water temperature, light, nutrition). Bermuda maximum air temperature⁸ (1891–1966) and air pressure⁹ (1880–1952) were considered the best available reflections of these influences. Water temperature is known to be well approximated by the former as shown by good agreement with short term recorded data (from the Bermuda Biological station, St George's, West Bermuda) and negligible island effect on surrounding climate⁸, while light levels are hypothesised to be correlated with high pressure—which presumably makes for good weather and sunny days.

A 13-yr running average was applied to each series (Fig. 1a–c) to reduce the importance of poorly known short term coral growth dynamics¹⁰, remove uncertainty in exact seasonal placement of band boundaries^{5,11}, and enhance long term climate fluctuations and coral response while minimising more random short term events. The overlap requirement for the three time series and the averaging procedure (which required dropping of six points at the ends) gave a 36-yr regression period (1946–11). When the coral band width chronology ($C(t)$) is regressed against maximal air temperature ($T(t)$) and air pressure ($P(t)$) (each series in index form), the result is

$$C(t) = -6.11T(t) + 3.12P(t) + 3.91$$

(significant by F test beyond the 0.01 level). The s.e. for the regression coefficient are 0.48 and 0.43 respectively. The multiple correlation coefficient $r=0.938$ (significantly different from zero beyond 0.01 level), so that the two variables explain 88% of the coral variance. The incremental contributions are 68% and 20% respectively. Figure 1a shows the coral series used in the regression and the coral series as reconstructed from the above equation. The fit, while not perfect, is remarkably good and comparable in precision with fits of weather¹² and other¹³ data obtained from tree-ring studies. The positive sign of the pressure coefficient reflects the expected relation of the corals to sunlight. The negative temperature coefficient, indicates increased growth (over the long run) during periods of lower than average water temperature. Although at first surprising, this becomes reasonable in the light of increased nutrient availability to corals during annual cold water overturn events¹⁴. The two coefficients thus offer a possible partial explanation of Bermuda coral growth: a negative relation to water temperature, which is actually a positive response to nutrient supply, and a positive relation to pressure, which may indicate a positive response to sunlight.

While the above results are useful in coral physiology studies, we would like to emphasise their utility in climate research where it is necessary to rely on historical weather records, often limited in extent, and where the introduction of information about additional climate variables or additional spatial locations would be particularly desirable. Fortuitous agreement can never be strictly excluded without a detailed causal model of coral–climate interactions; how-

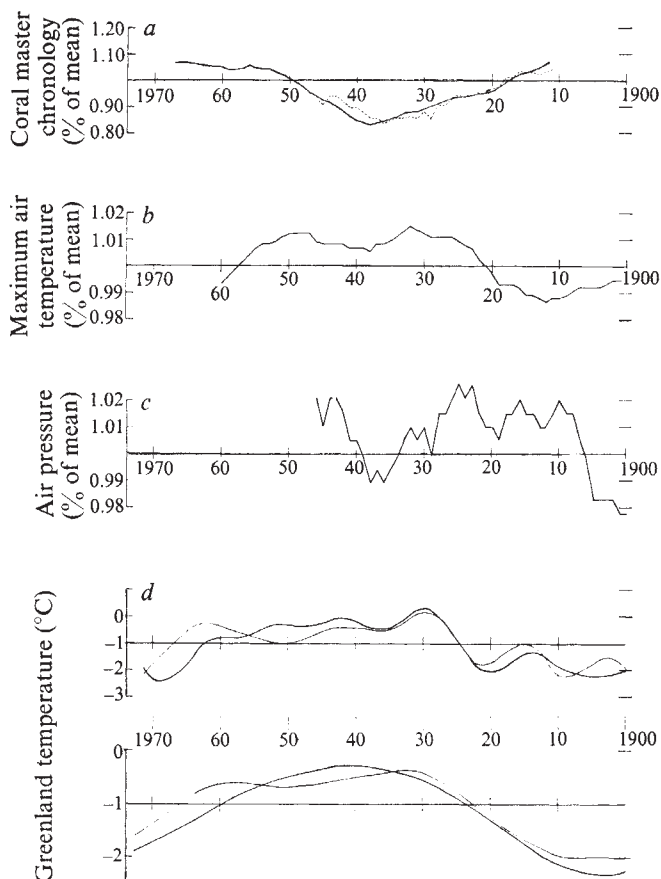


Fig. 1 a–c, Bermuda coral and climate time series smoothed by 13-yr running average. Data are presented as index values (each datum as a percentage of the particular series mean). The dashed line in Fig. 1a represents the coral series as reconstructed from the regression equation. Mean coral band width = 0.326 cm; mean maximum air temperature = 23.7 °C; mean air pressure = 18.7 mbar (+1,000 mbar) at sea level. d, From the data of Dansgaard *et al.*¹⁵ Greenland temperature smoothed by 10 yr (top) and 30 yr (bottom) low pass digital filter. Climate changes are not simultaneous on the west coast (heavy line) and east coast (thin line).

ever, the above results are statistically highly significant by conventional standards^{11,13}, and so offer the possibility of reconstructing additional palaeoclimatic variables from a variety of shallow oceanic locations. Since Bermuda maximum air temperature variations correspond well to global temperature variations¹⁵ (see Fig. 1d), and since global palaeotemperatures are becoming increasingly common and refined through oxygen isotope and palaeontological data, a long coral growth series may be used to obtain other important variables, in particular palaeobarometric pressure. This information becomes available in oceanic areas where other biological sensors (for example tree rings) are not available. The length of the chronology of band widths may be greatly extended in time by sampling larger coral heads on the Bermuda platform and by cross-dating older fossilised heads which compose the reef framework¹⁶.

Elsewhere long series of coral bands may be obtained at various past intervals (for example raised Pleistocene reefs) where, although overlap with the recent data may be impossible, fluctuations of past climate may be reconstructed. In addition to their intrinsic value, long series of palaeovariations or their variations are potentially useful to meteorologists and climatologists who wish to incorporate boundary conditions for starting and running models or to test or verify models by comparing model prediction with reconstructed data.

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Intelligence tests may favour the majority groups in a population

DISPUTE over the observed racial differences in intelligence has centred on causal attribution: cultural bias in the testing, hereditary disparities between races and environmental variation. All systematic interpretations of these differences, however, rest on the same fundamental psychometric assumptions. We here examine one of those assumptions.

We have previously suggested that the existence of genetic environmental interactions¹ could affect item selection in the construction of intelligence tests using interaction in its precise technical sense in the analysis of variance. Most other available data disagree², but are correlational rather than experimental in nature. The correlation methods used have been suitable for detecting only certain forms of interaction: the mainstream of psychological thought on testing is strongly inclined to continuous models, which do not readily suggest nor encompass many kinds of interactions³. On the other hand, one could argue from genetic theory⁴ that the number of possible interactions is so nearly infinite that no useful generalisations could result from pursuing such diversity. There has been almost no systematic attention to genotype-environment interactions in behavioural research or theory⁵. We here demonstrate that such interactions do in fact affect item selection and that these effects in turn contribute to the phenomena of group differences.

To reproduce minority group conditions several independent heterogeneous populations were formed from homogeneous groups, and intelligence tests were developed for each such base population using standard procedures. The characteristic performance of each group on each of these tests was then examined with reference to the representation of that group in the base population from which the test was derived. Hereafter 'test' refers to a test resulting from these test development procedures. 'Test base population' refers to the collection of individuals on which test development was based. 'Group'

refers to a homogeneous group identifiable by common background. 'Level' refers to the level or extent of representation in the test base population.

The experimental plan was a Latin square of 6 rows of test base populations and 6 columns of levels of representation, with random cell assignments of 6 groups, subject to the constraints of Latin squares. The six levels of representation were 22, 15, 11, 8, 5, and 0 individuals. Groups were six homogeneous lines of rats randomly chosen from the writer's collection, for which detailed genetic and environmental conditions have been reported elsewhere¹. The Latin square design, of course, provides that groups are randomly assigned in such a manner that each group appears once in each test base population and once at each level of representation. For the test development phase, individual animals were assigned on the basis of the Latin square. This established six independent test base populations each consisting of 61 individuals from each of the six groups at each of the six levels. To study characteristic performances, samples of 20 to 31 individuals were drawn from each of the six groups. Assignments of individual animals within the plan was random and blind: these were made by computer at the time of final data analysis.

Problems consisted of 19 geometric configurations of an extension of the Hebb-Williams maze^{6,7}, the conventional intelligence test for this species. Floors were either white or black, with partitions either of the same or contrasting colour. Thus four mazes were used, geometrically equivalent but differing visually; black on black, white on white, black on white, and white on black. One geometric configuration on one maze constituted one problem. Testing was at the rate of one problem a day with 15 trials per problem. Each geometric configuration was used in fixed order for four successive days, that is, over the four mazes, with order assigned at random for each individual for each configuration. Five measures were taken on each problem: mean start delay, mean exit delay, mean run time, trials to criterion, and number of errors. In all, 380 performance measures or item scores were obtained.

For the test development phase standardised item scores and test scores (the total of item scores) were used. Standardisation was based on the pooled data for all individuals. Correlations were obtained between item scores and test scores on each of the six test base populations independently. The 50 items showing highest correlation with the test scores for each test base population were then selected as an intelligence test for that population. This is, of course, the conventional procedure for item selection in test construction and development.

For the characteristic performance phase each of the resulting six tests was then 'administered' to the individuals in the characteristic performance samples drawn from each of the six groups. For each group independently this provided a mean or characteristic performance score on each of six different tests. From the Latin square each group was represented at a different level in each of the six test base populations from which the tests were derived. For each group both the characteristic performance means on the six tests and the level of representation of that group in the corresponding test base populations were ranked. For each group the rank difference correlation between rankings shows the relationship between group means and level of representation in the base population. The average of the six correlations was 0.562. The mean of the corresponding normal deviates from the tabled exact distribution yielded $z = 3.33$, $P < 0.001$ (ref. 8).

The mean characteristic performance of homogeneous groups was found to vary directly with the relative numbers of that group represented in the base population from which the test was derived. Therefore, there is a test bias inversely proportional to representation in the base population.

The results confirm the interaction hypothesis, and, moreover, it is now clear that genetic-environmental interactions do indeed affect item selection under the procedures commonly used in test construction. It should be emphasised, however, that these results and their implications stand independently of any

heredity-environment controversies. The psychometric implications would be identical had the homogeneous group differentiation been based on controlled environmental rather than genetic variation. Indeed, it seems reasonable to expect subsequent research will demonstrate interactions also occur with such environmental differentiation.

Finally, generalisation from these data to man is direct and not analogical: the experiment was an empirical test of common psychometric assumptions and procedures. Generalisation is therefore to those assumptions and procedures. The implications are far ranging. Majorities will score higher than minorities as a general artefact of test construction procedures. Theoretical approaches which ignore the existence of genetic-environment interactions ignore a significant source of individual and of group variation.

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Radiocarbon date for the largest extinct bird

IN December 1973 two fragments of an eggshell of the extinct giant elephant bird, *Aepyornis* sp., were obtained from a site 50 km west south-west of Fort Dauphin (21°1'S, 47°E), southern Madagascar. The bird, similar in appearance to, but not directly related to, the ostrich (*Struthio camelus*) was the largest ever to exist; the largest species had a height of 3 m, and laid eggs up to 35 cm long¹. The site from which the small fragments were obtained is located in coastal sand dunes some 100 m from the shoreline. The immediate region is underlain with granitic rocks capped by igneous detritus. The climate is semi-arid with a mean annual rainfall of 500-600 mm. The vegetation consists of bush interspersed with many endemic succulents including the very tall, stalk-like plants of the Didiraceae family².

We have obtained a radiocarbon date from one of the shell fragments, about 4 mm thick and weighing 11.78 g. To remove surface contamination the outer layer of the shell was removed with acid. The interior of the shell was then dissolved in hydrochloric acid and the resulting CO₂ carefully purified to exclude electronegative impurities. The ¹⁴C/¹²C ratio was determined in a CO₂ proportional counter to a statistical accuracy of 1σ standard deviation. An aliquot was analysed in a mass spectrometer, and its ¹⁴C/¹²C isotopic ratio was found to be -14.44‰ with respect to Craig's PDB standard. The radiocarbon age was calculated to be 1,000 ± 150 yr (UCLA-1893), which includes a correction for isotopic fractionation.

Tree-ring calibration of this date shows that the true age lies well within the margin of error³. Other egg shells have been dated using radiocarbon determination and have been found to give reliable dates⁴. Little contamination is to be expected from the arid environment containing little carbonate, except for that from sea shells in the sand dunes. Moreover, the removal of the outer shell layers of the *Aepyornis* shell fragment reasonably ensures that the date is not significantly in error.

An egg fragment found in sand dunes 3 km north-east of the village of Irodo, 45 km south-east of Diego-Suarez (12°19'S, 49°17'E) on the northern tip of Madagascar, has been radiocarbon dated at 1,150 ± 90 yr old, and nearby, an archaeological site has been dated at 1,090 ± 90 yr (Gak-692), (refs 5 and 6). Moreover, a number of egg fragments collected in locations around southern Madagascar have been dated at 1,970 ± 90, 2,930 ± 85 and 5,210 ± 140 yr old^{7,8}. It is especially noteworthy that in the archaeological site of Talaky on the southern coast of Madagascar (25°19'S, 45°30'E) *Aepyornis* egg shell fragments were found mixed in with pottery and other artefacts, including iron fragments. This site has been dated at 840 ± 80 yr (Gak-276). A historic reference by Flacourt⁹ even implies that these large birds may have existed in remote areas of Madagascar as late as the 17th century. Other archaeological sites are known to contain undated *Aepyornis* egg shell fragments.

The radiocarbon date presented here is of essentially the same period as an egg from the extreme north, suggesting that these birds were still widespread as late as the 10th century AD. It is thought that man entered Madagascar only as late as the middle of the first millennium AD¹¹ and it is possible that the fate of the giant bird population is tied to the late arrival of man. Yet the extinction of the elephant birds apparently occurred over many centuries and not suddenly. It is uncertain to what extent forest clearing played a major role in denying the birds a protective habitat.

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Scorpionflies as kleptoparasites of web-building spiders

ONLY a few species of spiders and insects are known regularly to inhabit spider webs. Known associations involve predation or commensalism. Spiders of the family Mimetidae invade the webs of other spiders to devour the owner of the web. Small spiders of the genus *Argyrodes* (Theridiidae) are often seen in the webs of larger spiders, where they seem to consume only small insects ignored by the spiders that constructed the web; this relationship is thus commensalism and not parasitism². The insect *Ranzovius* (Hemiptera: Miridae) seems to be a commensal in the webs of certain spiders³; however, *R. fennahi* may be predaceous on spider eggs⁴. The tropical hummingbird, *Phaethornis superciliosus*, has been observed feeding on insects

Table 1 Observed kleptoparasitism of spiders by panorpas

Panorpa sp.	No. of webs parasitised						Unknown Spiders	Row totals
	<i>Agelenopsis</i> Agelenidae	<i>Theridion frondeum</i> Theridiidae	<i>Mangora ornata</i> Araneidae	<i>Leucauge venusta</i> Tetragnathidae	<i>Micrathena gracilis</i> Araneidae	<i>Argiope aurantia</i> Araneidae		
<i>banksi</i>	1	3	8	4	5	1	3	25
<i>claripennis</i>	0	2	5	2	6	0	6	21
<i>debilis</i>	0	2	6	9	10	1	2	30
<i>helena</i>	2	0	3	2	3	0	0	10
<i>latipennis</i>	0	0	2	2	1	0	4	9
<i>maculosa</i>	0	0	1	0	1	0	0	2
<i>mirabilis</i>	2	1	7	4	4	0	10	27
<i>nebulosa</i>	0	0	5	4	1	0	5	15
<i>submaculosa</i>	0	0	4	3	3	2	2	14
Column totals	5	8	41	30	33	4	32	153

Percentage kleptoparasitic feeding by each species (number of observations of feeding in spider webs per total number of observations of feeding on dead insects) was: *P. banksi*, 30.1; *P. claripennis*, 29.2; *P. debilis*, 30.3; *P. helena*, 15.6; *P. latipennis*, 11.7; *P. maculosa*, 4.77; *P. mirabilis*, 31.0; *P. nebulosa*, 36.6; *P. submaculosa*, 22.2. Percentage kleptoparasitic feeding for all species combined was 24.6%.

trapped in webs of *Nephila clavipes* (Araneidae). The hummingbird–*Nephila* association may be parasitism because although only very small insects are taken by hummingbirds, they may remove a portion of the host's web for nest material⁵. I report here the kleptoparasitism of the prey of web-building spiders by scorpionflies of the genus *Panorpa* (Panorpidae), and I consider a likely selective context for the evolution of this unusual feeding strategy in scorpionflies. Field observations were conducted in south-eastern Michigan near Ann Arbor during 1971–74 (ref. 6).

Panorpas are scavengers, feeding primarily on soft-bodied dead or moribund insects, but occasionally eating living slugs, vertebrate carrion and pollen. Dead insects comprised between 89 and 97% of the diets of the nine species of *Panorpa* that occur in south-eastern Michigan. Diptera made up 47–69% of the dead insects in the diets of the species. A total of 675 feeding observations on the following nine species of *Panorpa* were recorded: *banksi* (86 observations), *claripennis* (75), *debilis* (102), *helena* (67), *latipennis* (87), *maculosa* (45), *mirabilis* (98), *nebulosa* (44), *submaculosa* (71). (See ref. 3 for details of food items and feeding behaviour of panorpas.) Panorpas are true kleptoparasites of web-building spiders, that is they enter webs and feed on trapped insects as well as on insects swathed in silk that are potential food for the host (Fig. 1). Kleptoparasitic behaviour in *Panorpa* spp. was observed frequently (Table 1). The data in Table 1 do not necessarily show that some *Panorpa* species feed in webs more than others, because various amounts of time were given to observation of the different species. Panorpas were observed feeding primarily in the webs of *Mangora ornata*, *Micrathena gracilis* and *Leucauge venusta*. These species seemed to be the most abundant in the woods where observations were made, which may account for

the large number of observations of panorpas parasitising these particular spiders.

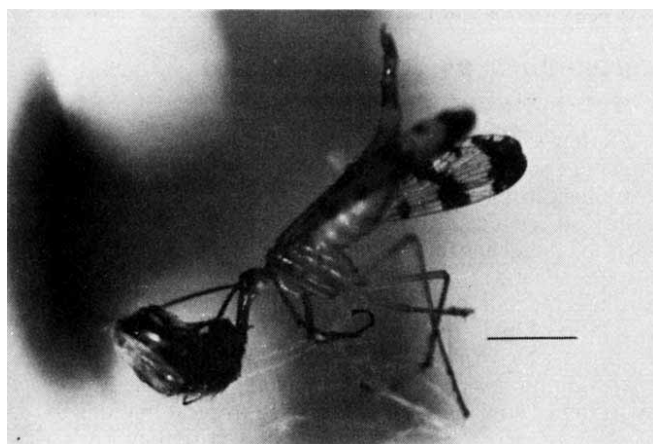
A panorpa, when it locates a spider web, may either fly directly to and land on an trapped insect, or walk into the web from the adjacent vegetation. Panorpas usually feed in the web and only leave it when approached by a spider; however, *P. banksi* on three occasions, and *P. helena* on one occasion, removed muscoid flies from the webs of *M. gracilis* and flew out of the webs with the flies in their mandibles. Panorpas move around in spider webs with little difficulty, though occasionally they may become entangled temporarily. If they cannot pull themselves free, they regurgitate a brown enteric fluid on to the portion of the web trapping their appendage(s). This fluid originates in the midgut and is similar in appearance to the tobacco juice of grasshoppers. Visual observations revealed that the webbing that traps panorpas dissolves when flooded with this secretion. Five *P. submaculosa* and five *P. banksi* were artificially entangled in the webs of *M. gracilis* in the laboratory to determine how rapidly they could free themselves; they escaped from the webs after 10 s–2 min ($\bar{X}$ = 1.1 min). Seven milked *P. banksi* (hand-held until they no longer regurgitated on my fingers) were unable to escape subsequent entanglement in webs of *M. gracilis* after 30 min.

Panorpas readily enter webs occupied by spiders. *Mangora* spiders are usually too small to capture and kill panorpas; however, *M. gracilis*, *L. venusta*, *Argiope aurantia* and some of the undetermined spiders (families Araneidae, Dictynidae and Theridiidae) do prey on panorpas. Fifty-nine per cent (53 observations) of the mortality of panorpas observed during 1971–74 was by web-building spiders. All the nine spiders of *Panorpa* studied, except the very large and aggressive *P. mirabilis*, were found in spider webs. The remains of equal numbers of male and female panorpas were seen in webs⁶.

When a panorpa feeding in a web is approached by the web owner, it may fly or drop from the web, or it may behave aggressively toward the spider. *Panorpa* males are armed with an enlarged genital bulb terminating the mobile abdomen, which is used in aggression towards spiders as well as towards other panorpas encountered around food. When a spider is struck by this genital bulb is usually stops briefly and may retreat. A *Panorpa* female, on the other hand, has no bulbous external genitalia, but may strike a spider with her abdomen, or butt it with her head. Aggressive behaviour of panorpas usually deters attacks by the small spider *M. ornata* and by the larger *L. venusta* and *M. gracilis*. Six *P. submaculosa* (three males and three females) and five *P. banksi* males were observed to act aggressively towards attacking *M. gracilis* in nature. The spiders were deterred and the panorpas escaped.

When a panorpa is seized by a spider, the panorpa regurgitates the same brown enteric fluid mentioned earlier and attempts to smear this on the predator. When applied by panorpas, the fluid was found to be an effective deterrent against the web-building spiders tested (*M. ornata*, *L. venusta*

Fig. 1 Female of *Panorpa debilis* feeding on an ensnared blowfly while hanging in the web of *Steatoda grossa*. Reference bar equals 5 mm.



and *M. gracilis*). Ten individuals of *P. debilis* were used to test the effectiveness of the enteric fluid against predation by spiders. The panorpas were introduced into pint jars containing spiders which had been starved for 3 d. Only two of the ten panorpas received eventually fatal injuries from the bite of a spider. Eight milked individuals of *P. debilis* were similarly tested and all fell prey to the spiders. When spiders are dabbed with the enteric fluid by panorpas they desist and begin cleaning themselves. The enteric fluid applied by panorpas is also effective against wolf spiders, crab spiders, jumping spiders and carpenter ants. The oral effluent of saw-flies, which consists of terpenoid resins sequestered from the host plant, is an effective deterrent against insect and spider predators⁷. The brown fluid regurgitated from the crop by grasshoppers also seems to have a protective function⁸.

Scorpionflies are common over much of eastern North America and are typically inhabitants of the herb strata of moist deciduous forests. In such habitats they may be the most abundant large insect. Adults of several species of *Panorpa* may occur together, often in large numbers, in the same woods. A 3-yr study of nine species of *Panorpa* in south-eastern Michigan reveals that the species overlap almost completely in their feeding ecology and that food is a limiting resource for these insects⁶. The species are not separated with respect to habitat utilisation, feeding location or time, or food type, however, the timing of adult seasonal emergence reduces overlap among the species. The *Panorpa* species investigated compete primarily in an interference manner (aggression) through intense intra- and interspecific aggression around food. Panorpas also share food with certain other arthropods (for example phalangids and scavenger beetles). Interference competition for food in panorpas influences the fitness of the competing individuals, since increased competition in panorpas results in reduced adult longevity under natural conditions. The reduction in longevity is a result of increased dispersal and movement into less desirable and more risky habitats in terms of exposure to predators. A selective history involving intense competition for food could account for the evolution of the dangerous strategy of kleptoparasitism of web-building spiders by panorpas.

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Natural daylengths regulate insect seasonality by two mechanisms

FOR many animal species in temperate regions of the world, the photoperiod is the major environmental factor that reliably signals forthcoming seasonal changes. With the exception of studies dealing with several species of birds^{1,2}, experiments generally have not shown what features of the natural photoperiod are used by animals to perceive calendar time. Not only have investigations of photoperiodicity usually used stationary photoperiods in the laboratory, but generally they have not taken into account the seasonal variations in the rate of change in the length of the day³;

these changes are greatest at the time of the equinoxes and smallest at the time of the solstices. Our results demonstrate for the first time that insects have evolved at least two mechanisms based on seasonal changes in photoperiod to end diapause at the end of winter.

We investigated three possible mechanisms for this. First, we considered whether the insects perceive day (or night) length as being either long or short; that is, day (or night) lengths shorter than a critical photoperiod elicit one response, and days (or nights) longer than a critical photoperiod elicit an alternative response. For example, in some insect species one or two critical photoperiods control diapause induction³⁻⁶. There is, however, evidence from only two species, *Nemobius yezoensis* Shiraki⁷ and *Wyeomia smithii* (Coquillett)⁸, that diapause termination is also controlled by critical photoperiods.

Second, we considered whether the insects perceive the actual duration of photophases (or scotophases) and show a quantitative (graded) response to seasonally changing daylengths. One insect species, *Chrysopa carnea* Stephens, has been shown to maintain diapause primarily through a quantitative response to the absolute duration of the shortening autumnal daylength and the very short days of early winter⁹. Quantitative responses to the duration of stationary daily photoperiods have also been shown in the laboratory for Odonata^{10,11} and for Diptera¹². There is no evidence, however, that this mechanism is used by insects to terminate diapause.

Third, we considered whether the insects perceive and respond to the direction of daylength changes *per se*, regardless of the actual daylength. Although *C. carnea* can perceive and respond to the direction of large daylength changes in the laboratory¹³, it has not been shown conclusively that insects react to the direction of naturally changing daylength.

Our results, derived from experiments with two species of green lacewings (Neuroptera: Chrysopidae), show that in nature, insects use at least the first two of the above three mechanisms in timing the end of their winter dormancy. In one species, *Meleoma signoretti* Fitch, days (or nights) are perceived as either long or short, and diapause ends when late winter days exceed a critical length (the first mechanism). In the other species, *Chrysopa downesi* Banks, diapause terminates at a rate directly related to lengthening days (the second mechanism).

Both of these lacewing species are, in nature, univoltine (they have one generation per year) and they spend a large portion of their annual cycle in an aestival-autumnal-hibernal diapause which is induced, maintained, and terminated primarily by photoperiod¹⁴. Therefore, to determine the type of photoperiodic stimuli that end diapause, we sampled natural populations from late autumn through to early spring. All test animals were F₁ offspring of females collected at Ithaca, New York. Individuals were reared and maintained outside according to methods given previously¹⁵. At each sample date we transferred 10-20 individuals into each of several stationary photoperiodic regimes and into a heated greenhouse under natural illumination (Tables 1 and 2).

The results (Table 1) for *M. signoretti* show that: first, diapausing larvae transferred to long day regimes in the laboratory terminate diapause within a relatively narrow time period, or they remain in diapause until death, if transferred to short day regimes. Second, natural daylengths shorter than a critical photoperiod maintain diapause during autumn and most of winter. Third, near the end of winter, natural daylengths which exceed the critical photoperiod provide the primary stimulus for diapause termination. Fourth, although the intensity of diapause decreases as the season progresses, the critical photoperiod does not change substantially, and the insects' response to photoperiod ends abruptly after diapause terminates, and fifth, the critical

Table 1 Median date of diapause termination (larval-pupal ecdysis) by *Meleoma signoretti* after transfer from out doors to various photoperiodic regimes

Date of transfer	10:14	12:12	Photoperiod (LD) 14:10	16:8	Natural
Nov. 22	— (0)	— (0)	Feb. 1 (10)	Feb. 1 (10)	Apr. 26 (2)
Dec. 22	— (0)	— (0)	Jan. 13 (11)	Jan. 31 (9)	Apr. 28 (5)
Jan. 22	— (0)	— (0)	Feb. 9 (10)	Feb. 9 (8)	May 1 (8)
Feb. 22	— (0)	— (0)	Mar. 5 (11)	Mar. 7 (12)	Apr. 20 (7)
Mar. 22	Mar. 30 (12)	Mar. 30 (12)	Mar. 30 (12)	Mar. 30 (12)	Mar. 31 (12)

Temperature: stationary photoperiods $24 \pm 1^\circ\text{C}$; natural daylength $23 \pm 4^\circ\text{C}$. No. of surviving individuals, from the original 12 to 14, in parentheses.

photoperiod for diapause termination in the laboratory is between light-dark (LD) 12:12 and LD 14:10. This range in photoperiods corresponds with the daylength (sunrise to sunset, plus twilight) in March, at the time diapause ends in nature.

The higher incidence of pupation in late season samples (Table 1) suggests that low temperatures enhance survival during diapause, especially at the end of winter when metabolic reserves are probably low. The approximately one-month delay in pupation in the November, December, January and February samples (Table 1), for natural daylength (in relation to the March sample) suggests that, although low temperatures are not necessary for ending diapause, they hasten diapause termination at the end of winter.

Table 2 Number of days (mean $\pm$ s.d.) to terminate diapause (initiate oviposition) by *Chrysopa downesi* after transfer from out-doors to various photoperiodic regimes

Date of transfer	11:13	12:12	Photoperiod (LD) 13:11	14:10	15:9	Natural*
Dec. 22	†	78 $\pm$ 12	22 $\pm$ 5	17 $\pm$ 5	14 $\pm$ 2	92 $\pm$ 10
Feb. 7	81 $\pm$ 12	46 $\pm$ 25	10 $\pm$ 3	—	8 $\pm$ 3	32 $\pm$ 5
Mar. 1	40 $\pm$ 34†	7 $\pm$ 1	7 $\pm$ 2	—	—	6 $\pm$ 1
Mar. 21	4 $\pm$ 2	4 $\pm$ 1	3 $\pm$ 1	—	—	4 $\pm$ 1

Temperature: stationary photoperiods $24 \pm 1^\circ\text{C}$; natural daylength $23 \pm 4^\circ\text{C}$. Number of pairs in each condition 8 to 10; incidence of oviposition in each condition 80 to 100%.

*Median dates of diapause termination in the December 22 to March 21 samples are April 5, March 20, March 8 and March 25 respectively.

†Diapause had broken in two animals in this sample.

‡Death occurred before diapause termination.

In contrast to those with *M. signoretti*, the tests (Table 2) with *C. downesi* show that: first, diapausing adults transferred into the laboratory after the winter solstice exhibit a quantitative response to daylengths that lie between LD 11:13 and LD 15:9, that is in the December 22 and February 7 samples the rate at which diapause comes to an end is directly related to the actual duration of daylength. Second, diapause maintenance after the winter solstice is related to the actual duration of the short winter days. Third, diapause is gradually brought to an end by the lengthening late winter days, that is, as the days lengthen there is an increase in the rate of diapause development, and diapause ends near the time of the vernal equinox (between March 1 and March 21), and fourth, diminution of both diapause intensity and sensitivity to photoperiod, which occurs slowly at the beginning of winter and quickly at the end of winter, is directly related to actual daylength.

Thus the data indicate that *C. downesi* and *M. signoretti* have evolved grossly different photoperiodic reactions that serve to synchronise the end of their diapause with the beginning of spring. In the case of *C. downesi* and other *Chrysopa* species for which we have considerable phenological data, the type of response is consistent with the phylogenetic relationships of the species¹⁶. For example, comparisons between populations of *C. downesi* and the closely related *C. carnea* from the north-eastern USA, show that both respond quantitatively to actual daylengths during diapause maintenance but each exhibits the response at a

different time of the year—autumn for *C. carnea* and early and late winter for *C. downesi*. As a result, these sympatric species, which readily hybridise in the laboratory and which hibernate in overlapping habitats, end diapause and begin postdiapause reproductive activity at least a month apart. This seasonal difference in the timing of reproduction helps prevent the univoltine *C. downesi* from hybridising with its close relative *C. carnea*. Therefore, the characteristic response of *C. downesi* to daylength during the winter not only serves to time the end of diapause with the advent of favourable spring conditions, but it also functions as an effective reproductive barrier which maintains this species as a separate entity in the regions of north-eastern USA and eastern Canada.

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Effect of light on egg-laying rate and mating speed in phototactic strains of *Drosophila*

IN *Drosophila*, phototactic behaviour can be quantitatively described using Hirsch-Hadler classification mazes¹. Flies entering the maze make a series of 15 consecutive light-dark choices and receive phototactic scores ranging from 1.0 (highly photonegative) to 16.0 (highly photopositive). A photoneutral population has an expected mean photoscore of 8.5. The phototaxis maze has provided a useful tool for the characterisation of phototactic behaviour in natural populations of *Drosophila* and for the creation of photopositive and photonegative strains of flies by artificial selection. Natural populations of *D. melanogaster* and *D. pseudoobscura* are photoneutral in general, but respond rapidly to selection for positive or negative phototactic behaviour^{2,4,7,8}. Genetic analysis of phototaxis in *Drosophila* suggests that this behaviour is a polygenic trait with low heritability^{3,4,6}. Suspension of artificial selection results in rapid reversion to photoneutrality which illustrates the principle of genetic homeostasis and suggests that changes

in fitness accompany selection for photopositive and photonegative behaviour⁸.

Two components of fitness—rate of egg deposition and mating speed—were measured in an unselected population and in a photopositive and photonegative population of *D. melanogaster*. The investigation was carried out under two sets of environmental conditions: continuous darkness and continuous light. Ten pairs of flies from each population were maintained separately for 1 week in continuous light and ten pairs from each population were maintained for 1 week in continuous darkness. Five replications were performed for each population in each condition. Egg-laying surfaces containing fresh cornmeal-molasses medium were replaced daily and the number of eggs deposited during the preceding 24 h was recorded.

Table 1 Average number of eggs laid by phototactic strains of *D. melanogaster* during 1 week of constant light and constant darkness

Population	Light (mean $\pm$ s.e.)	Dark (mean $\pm$ s.e.)	<i>t</i>
Unselected (7.9 $\pm$ 0.12)	1,111.2 $\pm$ 27.61	1,110.1 $\pm$ 34.76	0.25
Strain 0 (+) (13.93 $\pm$ 0.14)	748.2 $\pm$ 33.11	526.6 $\pm$ 25.26	5.32*
Strain 0 (–) (2.91 $\pm$ 0.11)	560.4 $\pm$ 36.17	1,672.7 $\pm$ 126.9	15.22†

* $P = 0.005$.

† $P = 0.0001$.

Results are shown in Table 1. The unselected strain was fairly photoneutral and the number of eggs deposited during 1 week did not differ in light or darkness. On the other hand, strain 0 (+) was highly photopositive and is seen to lay more eggs in continuous light than in a continuously dark environment. The opposite is true of flies from strain 0 (–), many more eggs being deposited when the flies were kept in continuous darkness.

To determine whether reduced insemination in light or dark was responsible for the reduction in oviposition, the effect of light and darkness on mating speed was investigated among flies from each population. Groups of twenty virgin males and twenty virgin females from each population were stored separately at $24 \pm 1^\circ\text{C}$ for 3 d. Males and females from the same population were then placed together in total darkness or light. After 5, 15, 30 and 60 min females were removed and placed individually in vials. At least five replications were made of each test. The presence of larvae in the vial indicated that the female had mated.

The proportion of flies mating at each time is shown in Fig. 1. In the unselected population, flies mated faster in the light. After 2 h (not shown) virtually 100% of the

females had mated. Flies from strain 0 (+) mated even faster in the light than did the unselected flies. At all four times, however, a greater proportion of flies from strain 0 (–) had mated in darkness than in the light. Early mating was definitely inhibited by the unpreferred environment but within 1 or 2 h females from all strains had mated both in light and in darkness. Therefore absence of insemination is not likely to be the cause of the alteration of oviposition rates in phototactic strains when placed in an unpreferred environment.

Table 2 Average number of eggs laid by phototactic strains of *D. pseudoobscura* during a week of constant light and constant darkness

Population	Light (mean $\pm$ s.e.)	Dark (mean $\pm$ s.e.)	<i>t</i>
Unselected (8.6 $\pm$ 0.19)	834.6 $\pm$ 112.94	880.6 $\pm$ 69.44	0.34
Strain 25 (14.93 $\pm$ 0.08)	1,641.0 $\pm$ 66.18	868.0 $\pm$ 115.14	5.82†
Strain 27 (2.54 $\pm$ 0.10)	1,346.6 $\pm$ 81.94	2,026.2 $\pm$ 152.35	3.92*

* $P = 0.005$.

† $P = 0.0005$.

The effect of light or dark on egg deposition was examined in a photopositive population (strain 25) and a photonegative population (strain 27) of *D. pseudoobscura* obtained from Professor Th. Dobzhansky. A recently collected population of *D. pseudoobscura* was provided by Dr F. Ayala. Egg deposition was measured in these three populations by the same method described for *D. melanogaster* (Table 2). Again, the presence or absence of light had no effect on the number of eggs laid by the unselected flies. Continuous light or darkness affected the photopositive and photonegative populations of *D. pseudoobscura* in the same way as observed for *D. melanogaster*.

It is difficult to know whether the preferred environmental condition (light or its absence) stimulates oviposition or if the unpreferred condition inhibits egg laying, or both. Regardless of the cause, it is obvious that the *Drosophila* species investigated here have a greater relative fitness in environments that they are genetically influenced to select.

Both *D. melanogaster* and *D. pseudoobscura* are light-independent with respect to mating behaviour⁹. Our results show that selection for phototactic behaviour may modify this light-independence in one direction or the other, at least for early matings. Photopositive and photonegative strains of *D. pseudoobscura* have been shown to mate with similar frequency in light, while in darkness, photonegative flies mate slightly more rapidly¹⁰.

Equally important to overall reproductive fitness is the

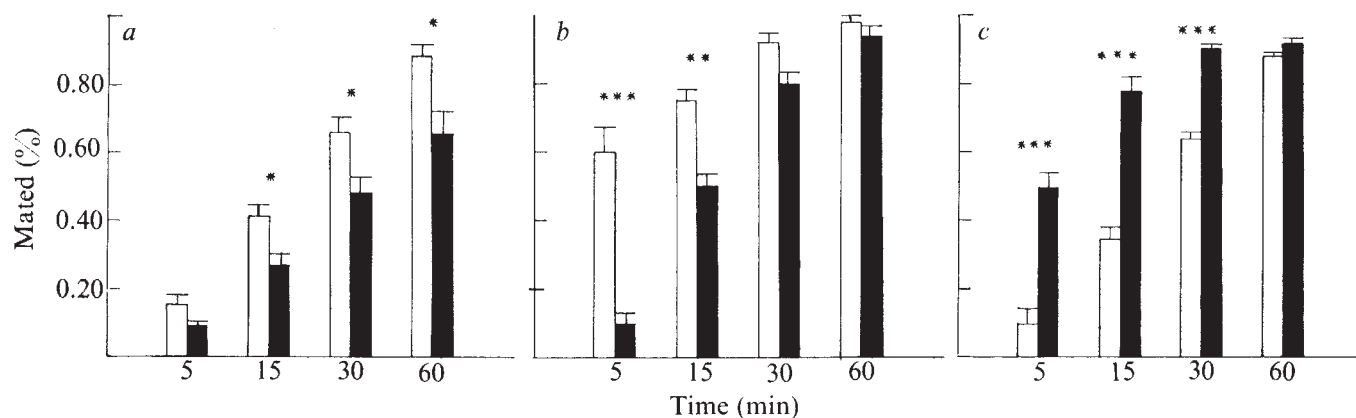


Fig. 1 Average percentage of females mated after 5, 15, 30 and 60 min in light (open bars) and darkness (solid bars). a, Unselected; b, strain 0 (+); c, strain 0 (–). * $P = 0.05$; ** $P = 0.01$; *** $P = 0.005$.

deposition of eggs after insemination. Individuals mating more slowly and depositing fewer eggs will leave fewer offspring relative to those individuals that mate and oviposit quickly. Light is an important environmental variable affecting natural populations. The presence of genetic variation for phototactic behaviour may be extremely important for adaptation of *Drosophila* populations to new or changing environments. In the experiments reported here, the light-dependent fitness of flies is directly related to their genotype for phototactic behaviour. Mayr¹¹ has proposed that behavioural changes precede morphological changes during evolution. The results presented here strongly support his idea.

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Corpus allatum activity and wing determination in *Megoura viciae*

ALARY polymorphism in aphids is influenced by several environmental stimuli acting prenatally and/or postnatally¹. In *Megoura viciae*, alatae are determined prenatally by exposing previously isolated apterae to a short crowding stimulus², and the physiological mechanism controlling this process would be expected also to show an immediate response to the stimulus. White³ suggested that the prenatal physiological control of alary polymorphism is exercised by the activity of the maternal corpus allatum (CA) which would produce less juvenile hormone (JH) when aphids are crowded than when they are isolated. She assumed that CA volume and nuclear diameter were directly proportional to JH synthesis and secretion and she reported that in *Brevicoryne brassicae* the CA nuclear diameter is greater in isolated aphids than in crowded aphids³.

haemolymph³. These studies have raised serious doubts about the role of JH in the control of alary polymorphism and the experiments reported here were carried out to determine the effect of crowding on the volume and ultrastructure of CA in apterous *M. viciae* in which alary polymorphism is controlled by a maternal physiological mechanism.

Isolated adult apterae, reared on 'tic' beans at $15 \pm 2^\circ\text{C}$ and a photoperiod of 16.5 h were prepared as alatae producers and apterae producers by confining them in specimen tubes either singly or in groups of 10 for 24 h (ref. 2). Aphids were fixed, embedded and stained for light and electron microscopy and the methods used are summarised in Table 1. Sagittal sections of whole aphids were cut serially and CA volume was estimated from planimetric measurements of consecutive sections in a series.

The mean CA volume in apterae producers ($22,804 \mu\text{m}^3$; s.d. $5,660 \mu\text{m}^3$; $n = 10$) is not significantly different from that of alatae producers ($19,028 \mu\text{m}^3$; s.d. $4,888 \mu\text{m}^3$; $n = 12$) ($P > 0.05$). Sagittal sections of the heads of three isolated and three crowded apterae were cut with glass knives. Thin sections were examined through an electron microscope (AEI model 6B) and the CA was identified with the aid of semithin sections ($0.05 \mu\text{m}$). Nuclei are irregularly shaped in both isolated and crowded apterae, they occur near the periphery of the gland, are often elongated and the nuclear membrane is highly invaginated. We therefore consider that nuclear dimensions cannot be used as a criterion of CA activity and that the contradictory descriptions of nuclear shape in active CA of other insect species^{10–13} make it impossible to use shape as an index for JH synthesis and secretion in *M. viciae*. Although mitochondria are usually larger in active CA than in inactive CA^{11,13}, there is no significant difference between the mean sizes of fifty mitochondria from each of the six aphids examined ($P > 0.05$). Smooth endoplasmic reticulum, membranous whorls and cytoplasmic vesicles, which are characteristic of inactive CA in some insect species^{14,15}, occur in both isolated and crowded apterae, and the cytoplasm also contains numerous neurosecretory axons.

The results indicate that the synthetic and secretory activity in CA of apterae-producing and alatae-producing aphids are the same, since the volume, mitochondrial size and cytoplasmic membrane composition are similar in both groups of aphids. Nuclear dimensions cannot be used as an index of CA activity, since nuclei have an irregular shape in adult apterae, and changes in the ultrastructure of the endoplasmic reticulum (which is the best individual index of CA activity) occur as much within a larval, pupal or imaginal instar as they do between instars^{14,15}. We therefore consider that differences in volumetric and nuclear dimensions between alatae producers and apterae producers and between alatiform and apteriform larvae can no longer be regarded as indicative of differences in CA activity^{3,4,16}. We can find no evidence to support either

Table 1 Summary of methods used in fixing, embedding and staining *M. viciae* for light and electron microscopy

	Fixative	Embedding material	Section thickness	Stain
Whole aphid	Aq. Bouin + 0.5% TCA ¹⁹	Celloidin and Paraplast	7 μm	Paraldehyde Fuchsin ¹⁹
Heads	Formaldehyde-glutaraldehyde ²⁰	Araldite	0.5 μm 60–120 nm	Toluidine blue Uranyl acetate + lead citrate

She also found that CA volume was greater in alatae, which have only apterous progeny, than in apterae which have alate and apterous progeny⁴. The application of JH analogues to alatiform larvae resulted in the apparent suppression of alate characters^{5,6}. Experiments with aphids, however, have shown that synthetic JH only initiates the production of supernumary larvae⁷ and that it has no apterising effect⁸. In locusts volume cannot be correlated with JH concentration in either CA or

White's³ assertion that the maternal CA is involved in the prenatal control of alary polymorphism or Elliott's¹⁶ suggestion that the CA is involved in the control of both ovarian growth and larval development in aphids. This does not mean that alary polymorphism is unaffected by JH concentration in the haemolymph since this may be controlled by the presence of specific metabolic enzymes¹⁷ and protein carriers¹⁸ which have been reported in other insect species.

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Manganese and photosynthetic oxygen evolution by algae

MANGANESE is required for the photoautotrophic growth of O_2 -evolving organisms, in which it functions in the O_2 -evolving complex of photosystem II. Mn-deficient cultures thus lose their O_2 -evolving capacity, but activity can be restored by reincorporation of Mn into the photosynthetic apparatus. The ratio of Mn atoms to chlorophyll molecules in O_2 -evolving organisms and preparations is 1:50–100 (ref. 1).

Heterocystous blue-green algae such as *Anabaena cylindrica* may provide a useful model for the study of the role of Mn in photosynthetic microorganisms since in cultures where all the cells are supplied with Mn, one cell type (the vegetative cell) evolves O_2 , whereas the other (the heterocyst), which arises from vegetative cells by differentiation, does not². We have therefore investigated the relationship between O_2 evolution and the Mn content of intact filaments and heterocysts of *A. cylindrica*, to determine whether the inability of heterocysts to evolve O_2 could be explained on a biochemical/biophysical basis by a lack of Mn. We grew the algae in the presence of ^{54}Mn , measured ^{54}Mn incorporation and related this to the O_2 -evolving capacity of various preparations.

Before using *A. cylindrica*, we carried out experiments on two other organisms. *Chlorella vulgaris* was used to demonstrate the correlation between the Mn content and

Table 2 Mn-chlorophyll ratio, O_2 -evolving capacity and photosystem I activity of *A. cylindrica* preparations

	Filaments	Heterocysts
Mn-chlorophyll ratio (g atoms:mol)	1:77	1:877
O_2 evolution ($\mu\text{mol per mg chl. per h}$)	97.3	0.0
Methylviologen supported photoreducing activity ($\mu\text{mol } O_2 \text{ consumed per mg chl. per h}$)	—	252*
AT^{32}P formation ($\mu\text{mol ATP formed per mg chl. per h}$)	—	64*

O_2 evolution was measured in a reaction mixture of 2 ml containing 30 mM HEPES, pH 7.5, and 2 mM $\text{K}_3\text{Fe}(\text{CN})_6$ as electron acceptor with the heterocysts. Methylviologen photoreduction was measured in a reaction mixture containing, in 3 ml of 30 mM HEPES buffer, pH 7.5: 2.5 mM NaN_3 ; 6.7 mM ascorbate; 5×10^{-5} M methylviologen, and 5×10^{-5} M dichlorophenol indophenol. AT^{32}P formation was measured in a reaction mixture containing, in 3 ml: 100 mM KCl; 6.7 mM ascorbate; pH 7.5, 30 mM HEPES; 6.7 mM phosphate containing $1-3 \times 10^6$ c.p.m. ^{32}P ; 2 mM MgCl_2 ; 0.05 mM phenazine methosulphate; 3.3 mM ADP. The ATP-trapping system contained hexokinase (Sigma type IV, 4 U) and 6 mM glucose. Experiments were carried out at a light intensity of $7 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$ at 25 °C, and samples contained 15–40 μg chlorophyll. —, Not measured. *Broken at 16,000 pound inch⁻² before assay.

the O_2 -evolving capacity of algal cells. Table 1 shows that intact cells and lamellar fragments of *C. vulgaris* both evolve O_2 , and in both cases the material is rich in Mn, with Mn-chlorophyll ratios of 1:26–33. The lamellar fragments, which have a somewhat lower capacity to evolve O_2 than the intact cells, have a slightly lower Mn content.

Intact spheroplasts of *Phormidium luridum*³, evolve O_2 but when a Mn-containing low molecular weight 'Hill factor' is released from broken spheroplasts, their O_2 -evolving capacity is lost. This latter capacity is restored when the factor is added back to the spheroplast preparations. Our data obtained with *P. luridum* (Table 1) provide quantitative support for the qualitative observation that the loss of the Mn-containing factor results in O_2 loss. They show that when O_2 -evolving spheroplasts which have a Mn-chlorophyll ratio very similar to that of intact filaments are ruptured to release the 'Hill factor', the loss of O_2 evolution is accompanied by a decrease in the Mn-chlorophyll ratio of the lamellar fragments from 1:62 to 1:592. Such results provide evidence that the Mn is associated with the O_2 -evolving reaction centre of photosystem II in blue-green algae.

In experiments with *A. cylindrica* (Cambridge Culture Collection No. 1403/2a) the alga was grown axenically in 2 l batch culture and ^{54}Mn (20 μCi) was added at the time of inoculation. After 8 d, when the algae were in late log phase of growth, the cells were collected by centrifugation, washed three times in HEPES buffer (30 mM, pH 7.5) containing 1 mM EDTA, and finally resuspended in HEPES buffer. Isolated heterocysts were prepared from such filaments by treatment with lysozyme (1 mg ml⁻¹) in mannitol-HEPES buffer (0.5 M and 30 mM respectively, pH 7.5) for

Table 1 Mn content and O_2 -evolving capacity of *C. vulgaris* and *P. luridum* preparations

Organism	Material	Mn-chlorophyll ratio (g atoms: mol)	O_2 evolution ($\mu\text{mol per mg chl. per h}$)
<i>C. vulgaris</i>	Intact cells	1:26	39
	Lamellar fragments	1:33	25
<i>P. luridum</i>	Intact filaments	1:51	107
	Spheroplasts	1:61	44
	Spheroplast fragments	1:592	4

C. vulgaris (Cambridge Collection No. 211/11h) was grown in 2 l batch culture in the medium of Goulding and Merrett⁴, at a light intensity of $4.5 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$ at 25 °C. The culture was bubbled with air+5% CO_2 (1.0 l min⁻¹). ^{54}Mn (10 $\mu\text{Ci l}^{-1}$) was added at the time of inoculation of the alga and the cells were collected in late log phase. Cells were then washed in 30 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer, pH 7.5+1 mM EDTA, then suspended in HEPES buffer and broken by treatment (twice) in a Yeda press at 1,500 pounds inch⁻². Lamellar fragments remained in the supernatant after centrifugation of the broken cells at 300g for 5 min, and were collected by pelleting at 30,000g for 15 min. O_2 evolution was measured using a Rank-type O_2 electrode in 2.0 ml of 30 mM HEPES buffer containing 2 mM $\text{K}_3\text{Fe}(\text{CN})_6$. *P. luridum* (Indiana University strain 426) was grown, preparations were obtained, and O_2 evolution was measured as before³. ^{54}Mn (10 $\mu\text{Ci l}^{-1}$) was added at the time of inoculation and the material was collected in late log phase of growth. Chlorophyll was measured according to Mackinney⁵. Each value is the mean of duplicate determinations.

30 min at 37 °C. The pellet was resuspended in HEPES buffer, treated twice in a Yeda press at 1,500 pounds inch⁻² under argon and diluted three to five times with HEPES buffer+1 mM EDTA. Heterocysts were then separated from vegetative cell material by differential centrifugation in HEPES buffer. Samples of the intact filaments and of isolated heterocysts were then assayed for a capacity to evolve O₂, for photosystem I activity and for ⁵⁴Mn content. O₂ evolution was measured using a Rank-type O₂ electrode; photosystem I-dependent electron transport was measured as O₂ consumption by photoreduced methylviologen when ascorbate+DCPIP was supplied, and cyclic photophosphorylation was measured as AT³²P formation according to the method of Avron⁴. ⁵⁴Mn incorporation was measured as radioactive γ -emission using a well-type scintillation counter (Panax type SC-LP).

Table 2 shows that the inability of isolated intact heterocysts to evolve O₂, noted before², is accompanied by a depletion of Mn, the content of which in isolated heterocysts is only 9% of that in the O₂-evolving whole filaments. Such a lack of O₂ evolution is not accompanied by a general loss of photosynthetic functions by the heterocysts since they can carry out photosystem I-mediated electron transport and cyclic photophosphorylation, and have a normal chlorophyll content.

Our results thus provide evidence that not only is Mn associated with the photosystem II reaction centre of blue-green algae, but that heterocysts of N₂-fixing algae are deficient in Mn and a functional photosystem II. This sophisticated mechanism and possibly the lack of certain photosystem II pigments⁵, ensures that in heterocysts there is no photosynthetically produced O₂ to inactivate the O₂-sensitive nitrogenase which occurs within these differentiated cells.

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Enzyme to break down lettuce endosperm cell wall during gibberellin- and light-induced germination

THE physiology of lettuce seed germination has been a subject of extensive study, but very little is known of the fundamental biochemical events which occur during germination. Here we report that the endosperm cell wall is largely composed of a mannose polymer (that is, a mannan); and that germinating seeds produce an enzyme capable of its breakdown.

The endosperm, a tissue two or three cell layers thick which completely surrounds the embryo, is a physical barrier which is ruptured by the growing radicle during normal lettuce seed germination. It has been proposed by some that the endosperm is degraded by the production of a cellulase before radicle protrusion can take place¹; others have maintained² that the germinating embryo itself develops sufficient thrust to penetrate the intact

endosperm. It has been demonstrated by microscopy that substantial degradation, in an ordered fashion, of the endosperm cell wall does occur during germination³. Whether this is a necessary event for germination (radicle protrusion) to take place is still not clear; nor has the biochemical mechanism of this degradation been established until now. An enzyme produced in germinating seeds and capable of degrading the endosperm cell wall has been sought since Ikuma and Thimann reported some success in detecting a carboxymethylcellulase and a pectinase⁴; and it has been shown recently that there are indeed changes in extractable carboxymethylcellulase activity during germination⁴.

One puzzling question arising out of the enzymic endosperm-degradation hypothesis is how the cell walls of the impinging radicle are themselves protected from degradation. Of the several possible explanations, perhaps the simplest is that there is some major difference in the composition of the radicle and the endosperm cell walls.

Accordingly, we have examined the total sugar composition of the cell walls of whole lettuce seeds, of dissected cotyledons, radicles and endosperm, and of 1-d-old seedlings (Table 1). This analysis shows that the endosperm, which contains two-thirds of the total seed cell wall polysaccharide material, has an unusual composition, comprising about 60% mannose. The cell walls of the cotyledons, radicles and the young seedling have considerably lower levels of mannose, and have a composition more typical of the cellulosic primary cell wall of higher plants⁵. The presence of mannose, initially identified by cochromatography with a mannose standard on gas-liquid chromatography, as the major sugar in the hydrolysate of endosperm cell walls was confirmed by both paper chromatography (ethyl acetate-pyridine-water, 8 : 2 : 1 v/v) and by the cysteine-sulphuric acid test¹⁰. The large amounts of mannose do not arise from a cytoplasmic storage polysaccharide (lettuce is not regarded as a carbohydrate-storing seed), since after homogenisation in ice-cold buffer, only 5% of the amount of polysaccharide precipitated at 3,000g is found in a 27,000g particulate fraction, where any storage bodies would be precipitated.

The occurrence of mannose in such a quantity in the endosperm cell wall means that a large portion of the cell wall polysaccharide is a mannan. This can be assumed to be a linear β -1,4 mannan (possibly a β -1,4 glucomannan, and perhaps with galactose side-chain substitution) on the basis of what is already known about other higher plant mannans¹¹. The cell-wall mannan could be a component of the microfibrillar element shown to be present in the thick ramified lettuce endosperm walls³. Partially microfibrillar mannans also form a large part of the thickened cell walls of vegetable ivory nut (*Tagua*) endosperm¹²; and in the context of lettuce endosperm cell wall mechanical properties, the occurrence of cell-wall mannans in such a seed endosperm, noted for its hardness and mechanical rigidity is interesting.

The lettuce endosperm cell wall is broken down during germination, so the presence of an endogenous enzyme which can decompose the major cell wall component was investigated. Table 2 presents the results of an experiment, incorporating a viscometric assay, which show that an enzyme activity occurs during germination which brings about a rapid degradation of locust bean (carob, *Ceratonia siliqua* L.) galactomannan, a polysaccharide which contains a β -1,4 mannan backbone chain¹¹. This is indicative that germinating lettuce seeds produce an endo- β -mannanase (EC 3.2.1.78). The presence of an α -galactosidase, which can also bring about galactomannan viscosity changes at the expense of a considerable substrate hydrolysis¹², is unlikely, since incubation of the most active extract with galactomannan for 3 h led to a less than 1%

Table 1 Percentage of sugar composition of lettuce seed tissue cell walls (mg per 100 mg carbohydrate)

	Seeds imbibed in darkness for 3 h				Seeds imbibed in light for 23 h	
	Endosperm	Coty-ledons	Radicle	Whole seeds	Endosperm	Seedlings (cotyledons and radicles)
Rhamnose	0.4	1.4	1.7	0.9	0.3	2.1
Fucose	0	0.6	0	0.3	0	1.1
Ribose*	0.5	2.9	5.2	2.3	0.7	3.7
Arabinose	9.5	18	15	16	11	17
Xylose	1.5	7.4	6.0	4.0	2.0	7.0
Mannose	58	4.8	7.4	39	53	3.7
Galactose	9.5	13	8.6	12	11	13
Glucose	10	18	28	13	12	23
Uronic Acids	10	34	28	12	10	29
mg Carbohydrate in hydrolysate per 50 seeds (~per 50 mg)	1.73	0.81	0.18	2.71	1.13	1.15

Batches of 50 lettuce seeds (*Lactuca sativa* var. Grand Rapids, Ferry Morse Seed Co. 1971) were imbibed for 3 h, or germinated in white light for 23 h. Embryos plus endosperm were separated from the surrounding layers and then dissected out under a dissecting microscope. The tissues were placed immediately in 70% ethanol and ground in a Micro-Dounce Homogenizer. The cell-wall material was collected by centrifugation at 3,000g for 10 min, and washed once with alcohol and twice with chloroform-methanol (2:1 v/v) then dried by rotary evaporation. This wall preparation was dissolved in 0.05 ml 72% w/w sulphuric acid for 3 h at room temperature, then diluted to 4% w/v with 1.4 ml water and hydrolysed in a sealed tube for 1 h at 120 °C. The hydrolysate was neutralised with barium carbonate and made up to 4.0 ml. Aliquots were assayed for total sugar⁶ and acidic sugars⁶, and analysed for neutral sugar composition by gas-liquid chromatography of the derivatised alditol acetates⁷, on a 2.7 m x 2 mm glass column (0.2% polyethyleneglycoladipate, 0.2% polyethyleneglycolsuccinate, 0.4% XF-1150 (ref. 8) on Chromosorb W, H.P., 80-100 mesh, (Chromatographic Specialties Ltd)) at 182 °C using helium carrier gas (30 ml min⁻¹) in a Hewlett-Packard 402 Gas Chromatograph. Neutral sugar composition was calculated from measured peak areas using correction factors previously established with sugar standards; total sugar composition was computed using determined neutral sugar composition and known different behaviour of individual standard sugars in each of the two assays.

*Presumably present as cytoplasmic contamination.

release of potential reducing power (assayed by the ferricyanide method¹⁴).

Endo- β -mannanase enzyme activity was seen both in light- and gibberellin-induced germination. Activity seems to be correlated with the degree of germination, as measured by radicle protrusion. Minimal activity was detected when the germination index was low (10 h) and none was found in seeds imbibed for only 1 h. It is not

wall is quite clear. If a cellulase is indeed produced in germinating lettuce seeds, it cannot by itself lead to appreciable degradation of the endosperm cell wall; it might contribute, or may have some other, as yet unclear, function. Since the composition of the endosperm cell wall does not change significantly over the first day of germination, while the total amount of cell wall polysaccharide present is decreased (Table 1) and the endosperm tissue

Table 2 Galactomannan breakdown by lettuce seed extracts

Treatment	Time after imbibition (h)	Units enzyme activity per 1 g seeds	% Germination
Darkness	1	0	0
	10	0	0
	13.5	2.6	1 $\pm$ 1
15 min red light after 2 h imbibition	10	4.8	1 $\pm$ 1
	13.5	25	77 $\pm$ 3
100 μ g ml ⁻¹ GA ₃ in darkness	10	0	
	13.5	9.6	17 $\pm$ 3

Batches (1g) of Grand Rapids lettuce seeds were incubated as shown above, ((9-cm Petri dish on two layers Whatman No.1 filter paper with 6 ml liquid at 23 °C), collected and ground in a total of 8 ml ice-cold McIlvaine's buffer, pH 5.0, and centrifuged at 17,000g for 10 min. This supernatant was fractionated by taking a 20-60% saturated ammonium sulphate cut¹⁵, resuspending in half-strength buffer, and dialysing overnight at 4 °C against tenth-strength buffer. After a final clearing centrifugation at 17,000g for 10 min, the extract was made up to 4.0 ml with tenth-strength buffer. Locust bean galactomannan (Sigma) was purified by dissolving in water and precipitation from solution with 80% ethanol (twice), redissolved and freeze dried (analysis: 70% mannose, 28.5% galactose, 1% ribose). Decrease in viscosity was measured in a Cannon-Ubbelohde suspended level viscometer at 30 °C. The assay mixture consisted of 10 ml 0.1% galactomannan (dissolved in tenth-strength buffer) and 1 ml of the lettuce seed extract. The rate of decline of viscosity in active fractions was linear over the first hour. The enzymic activity per 1 g seeds is shown, as well as the degree of germination (radicle protrusion) at the time of collection. A unit of activity is defined as that which will bring about a 1% decrease in viscosity in 30 min.

3-min boiled extract showed no activity.

possible to say yet whether enzyme production precedes or is necessary for radicle protrusion.

Endo- β -mannanases are known to be involved in the degradation of storage galactomannans in germinating legume seeds¹². Although the report elsewhere of carboxymethylcellulase activity detectable during the same early period in germinating lettuce seeds suggests that the endo- β -mannanase we have identified may have poor substrate specificity⁴; that "carboxymethylcellulase" has little or no available cellulose substrate in the endosperm cell

becomes much easier to homogenise, this suggests that other cell-wall degrading enzymes are present. These observations are consistent with the endosperm cell wall degradation reported by Jones³. Unfortunately, Jones did not specify whether degradation takes place over the entire endosperm, or is localised to one region, such as, for example, the micropylar end of the seed from which the germinating radicle emerges; this is a very important detail.

Such questions as when and where in the seed the

endo- β -mannanase is produced, and how this process is controlled, are of interest in the study both of lettuce seed germination and of the control of enzyme production in plants.

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Reduced sperm motility in the isthmus of the rabbit oviduct

SPERM transport through the female reproductive tract has been studied extensively in mammals^{1,2}, but no systematic evaluation of the motility of spermatozoa at different levels of the tract after coitus has been reported. We describe a procedure for collecting oviducal spermatozoa and present observations on an unexpected reduction in the proportion of motile spermatozoa in the isthmus of the rabbit oviduct over the 12-h interval between mating and fertilisation.

Thirty-six New Zealand White does were each mated with three or four bucks. Six does were killed at 1.5, 4, 6, 8, 10 and 12-h post coitus by intravenous overdose of sodium pentobarbitone (Nembutal, Abbott). Haemostats were clamped across the utero-tubal and isthmo-ampullary junctions of the oviduct and across the uterine horns above the cervixes. The uteri were removed by cutting across haemostats placed below those clamping the utero-tubal junctions. Uterine sperm were collected by aspiration, after injecting 0.5 ml of a 5% mixture of rabbit serum (previously stored at -20°C) in Tyrode's solution (pH 7.4) into each horn. To collect oviducal sperm, isthmic and ampullary segments of the oviduct were cannulated at their ovarian ends with 10 λ micropipettes (Yankee Disposable Micropipette, Clay Adams) introduced 1–2 mm into the lumen. Oviducal contents were flushed into the micropipettes by

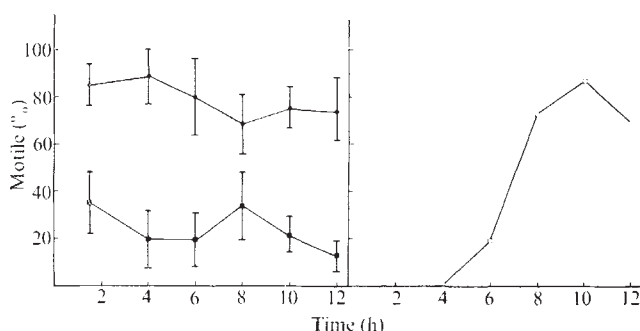


Fig. 1 Mean % of motile rabbit sperm $\pm$ s.d. recovered from uterine horns (●) and Fallopian tube isthmi (■) during 12 h post coitus; data based on sperm counts in samples from 12 uterine horns and tubal isthmi at each interval. The proportions of motile ampullary sperm (□) are calculated from the sum of motile and non-motile sperm recovered from 12 ampullae at each interval.

introducing the blunted needle of a Butterfly-23 Infusion Set (Abbott) 2–5 mm within each segment and injecting 10–20 λ of serum-Tyrode's. The tubing of the infusion set was mounted in a micropipette controller (Clinac Micropipetter, Lapine), which was used to regulate the volume and rate of fluid delivery.

Uterine and oviducal contents were examined immediately at room temperature by phase-contrast microscopy and sperm showing any evidence of flagellar movement were classified as motile: those lacking any activity were considered non-viable. All sperm were examined for the state of the membranes overlying the acrosomal region of the head (Table 1). The proportion of motile sperm in homogeneous uterine samples was estimated by counting 100–200 sperm from each horn; all sperm in ampullary samples were counted. Motile isthmic sperm flushed from 10- λ micropipettes were not distributed randomly on microscope slides, so they were counted in sets of 100 until the proportion of motile cells in successive sets varied by less than 5% from the percentage motile sperm in the cumulative total.

To exclude the possibility that estimates of isthmic sperm motility were biased by differential recovery of non-motile sperm, the contents of ten isthmi were collected in three serial flushes. The first flush of 10 λ was collected as described. The second was 0.5 ml of serum-Tyrode's, followed by 0.5 ml of 1% sodium dodecyl sulphate (SDS) in distilled water. These were collected directly in 0.5 ml polyethylene centrifuge tubes and sperm were concentrated by centrifugation for 30 s in a Beckman microcentrifuge (Model 152). Two isthmi at 4, 8 and 12 h post coitus and four isthmi at 6 h post coitus were flushed in this way. The mean percentage of motile spermatozoa in 10- λ and 0.5 ml serum-Tyrode's samples did not differ significantly ($22.1\% \pm 15.2$ as against $17.0\% \pm 10.0$; $P > 0.25$, $f = 0.0073$ (1,18) by one-way analysis of variance). The number of sperm recovered in 0.5 ml SDS flushes was less

Table 1 Motility and state of the head membranes of sperm recovered from the reproductive tract of female rabbits between mating and fertilisation

Hours post coitus	Uterus			Isthmus			Ampulla		
	No. counted	Motile* (%)	Non-motile† altered heads	No. counted	Motile* (%)	Non-motile† altered heads	No. counted	No. motile‡	No. non-motile altered heads
1.5	900	85.0 $\pm$ 8.8	7.8 $\pm$ 5.1	287	35.1 $\pm$ 13.0	36.0 $\pm$ 16.8	5	0	4
4	1,200	88.8 $\pm$ 11.6	5.6 $\pm$ 7.4	3,858	19.0 $\pm$ 12.1	55.9 $\pm$ 16.3	6	0	4
6	1,200	80.7 $\pm$ 15.9	11.6 $\pm$ 11.7	5,689	19.6 $\pm$ 10.5	68.2 $\pm$ 14.5	21	4	10
8	1,300	68.1 $\pm$ 13.2	12.2 $\pm$ 8.8	6,064	33.5 $\pm$ 14.3	50.2 $\pm$ 13.9	60	43	9
10	1,500	75.6 $\pm$ 8.6	10.5 $\pm$ 6.6	5,118	21.8 $\pm$ 6.9	48.6 $\pm$ 10.0	166	145	15
12	1,600	74.7 $\pm$ 13.0	13.9 $\pm$ 9.3	6,690	12.8 $\pm$ 6.1	66.3 $\pm$ 15.5	139	97	19

*Mean % $\pm$ s.d. of intact motile sperm recovered from 12 uterine horns and 12 Fallopian tube isthmi at each post coital interval.

†Mean % $\pm$ s.d. of non-motile sperm with lifted or absent acrosomal head membranes. The remainder of non-motile sperm recovered in uterine and isthmic samples either seemed to have intact head membranes, with phase-dense acrosomes, or were aggregated with leukocytes.

‡Ampullary sperm numbers are based on the total number of sperm recovered from all of 12 ampullae sampled at each post coital interval.

than 1% of the 2,000–4,000 previously estimated to be present in the isthmus at these times^{3–7}. These data support our assumption that the proportion of motile sperm in 10- λ samples is a valid representation of the motility of the total population recoverable from the isthmus in the first 12 h post coitus.

Seventy per cent or more, of uterine spermatozoa were motile at all collection times and most showed vigorous progressive movement (Table 1, Fig. 1). In contrast, the motility of isthmic sperm was uniformly poor, with few showing progressive movement and usually less than 35% any sign of flagellar activity. Motile ampullar sperm invariably showed vigorous motility of better quality than that of isthmic sperm from the same oviduct, and their motility often exceeded that of uterine sperm.

Most non-motile sperm throughout the female tract exhibited morphological changes in the membranes overlying the sperm head (Fig. 2). These changes varied from a lifting of the acrosomal cap to complete loss of the acrosome. Non-motile sperm with altered head membranes usually constituted less than 15% of all uterine and ampullar sperm, compared with 50%, or more, from the isthmus (Table 1). Motile sperm with

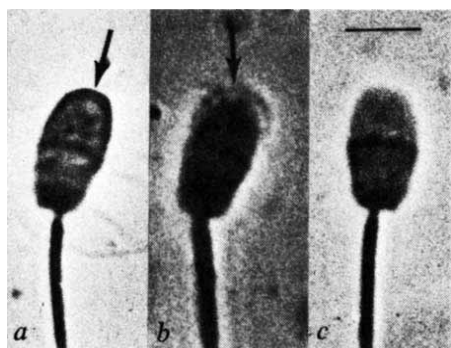


Fig. 2 Rabbit sperm collected 10 h post coitus from the isthmus of the Fallopian tube; phase-contrast microscopy. *a*, Sperm with intact, phase-dense acrosome (arrow at acrosome margin); *b*, non-motile sperm with altered head membranes (arrow on edge of expanded acrosomal cap); *c*, non-motile sperm with absent acrosomal cap. Calibration bar = 5 μ m; $\times 1,900$.

altered or missing acrosomal caps were not observed in uterine samples and were seen only rarely in isthmic samples. In comparison, motile ampullar sperm with lifted or missing acrosomal caps were seen frequently at 8 h post coitus and thereafter.

These findings are unexpected in the light of literature on sperm transport^{1,2} and capacitation^{8,9} in mammals, since it has been generally assumed that spermatozoa present throughout the oviduct before ovulation would be highly motile. The reduction in number of viable sperm in the isthmus seems to be a means of regulating sperm transport not previously appreciated. Together with the cervix and utero-tubal junction, the isthmus has been considered to restrict the passage of sperm to the site of fertilisation^{10,11}, but a physiological basis for its action was unknown. Whether the isthmus solely reduces the number of sperm entering the ampulla, to protect against polyspermic fertilisation^{10,11}, or whether removal of defective or incompetent sperm also occurs in this region¹² remains to be determined. Our finding that a high proportion of non-motile isthmic spermatozoa have disrupted or missing acrosomes, whereas similarly altered ampullar sperm are often motile, suggests that: (1) the adverse influence of the isthmic environment may be directed against the plasma membrane of the sperm head, and (2) spermatozoa remain viable in the isthmus by virtue of stable plasma membrane configurations. Such sperm may be considered to be a selected population. The virtual absence from the isthmus of motile spermatozoa with altered head membranes indicates that the stimulus for induction of specific acrosomal changes which precede fertilisation may be limited to the upper oviduct.

The reduced motility and altered morphology of isthmic sperm may depict their condition *in vivo*, or may be manifestations during recovery of other physiological changes undergone within the oviduct. Previous investigations of sperm transport in the female rabbit focused on the number of spermatozoa present in various regions of the tract^{3–7} and often used spermicidal detergents to aid recovery. Moreover, observations on the physiology of spermatozoa in the oviduct have usually involved direct transfer of uterine sperm to the ampulla, thus bypassing the utero-tubal junction and tubal isthmus. It is not surprising, therefore, that the phenomenon of reduced sperm viability in the isthmus and its implications for understanding sperm transport and capacitation in the female rabbit have gone unrecognised.

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Nuclear transplantation in the rabbit egg

SINCE the experiments of Briggs and King¹, in which nuclei from blastula cells were transplanted by microinjection into enucleated frogs' eggs, the technique of nuclear transplantation has been used extensively to study interactions between nucleus and cytoplasm during differentiation in amphibians^{2–5}. But severe difficulties are encountered in attempts to apply this technique to the mammalian egg. The rabbit egg, with a diameter of 100 μ m, is 1,000 times smaller in volume than a frog's egg, and the mouse egg has a volume one-third that of a rabbit's egg. An alternative, and technically easier method of transplanting a nucleus is by inducing fusion of an egg and a cell with Sendai virus. The technique of virus-induced fusion, originally developed for hybridising somatic cells^{6,7}, has been adapted to fuse somatic cells with unfertilised or fertilised mouse eggs^{8–13}. Although Sendai virus was shown not to harm mouse embryos in these experiments, and cells were successfully fused, the transplanted nuclei did not survive. Unfertilised mouse eggs which were fused with cells from embryos at the 2–8-cell stage failed to cleave⁸.

I report here experiments in which nuclei of embryonic cells were introduced into unfertilised rabbit eggs by both microinjection and virus-induced fusion. Unfertilised eggs were recovered from the Fallopian tubes of females induced to ovulate by a single intravenous injection of chorionic gonadotrophin (Chorulon, Intervet). In virus-induced fusion experiments which required denuded eggs the zona pellucida was removed mechanically.

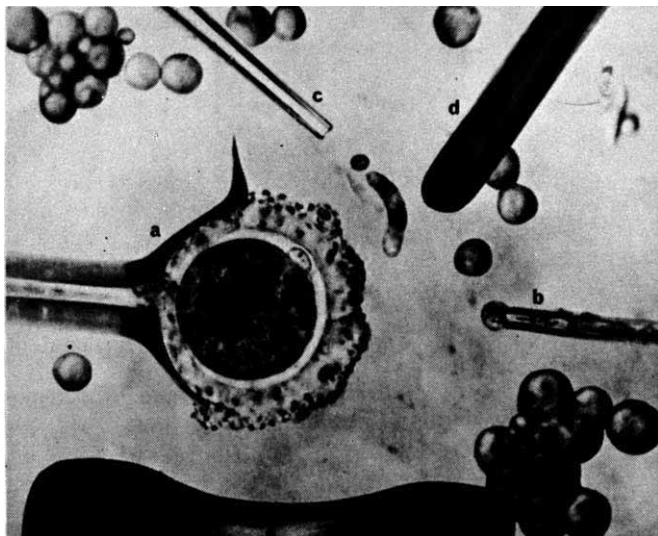


Fig. 1 General arrangement of instruments, egg and donor cells ($\times 300$). *a*, Cup, holding egg; the spike projecting from the cup is for removing sticky cytoplasm and corona cells from the tip of the injection micropipette. A single polar body is visible in the perivitelline space of the egg. *b*, Injection micropipette, containing a donor cell. *c*, 'Sausaging' pipette for deforming a donor cell into a cylinder before it is taken into the injection micropipette. *d*, A flame-polished needle for manipulating eggs and cells. *e*, Large bore take-up pipette for removing eggs after microinjection. During an experiment instruments, eggs and donor cells are in several focal planes; in this photograph they have been arranged in a single plane of focus for clarity of presentation.

Donor cells were obtained from morulae recovered from females mated 48 h previously. In the first series of experiments the donor nucleus was introduced into the egg at any stage of its cycle. In the second series the aim was to synchronise donor and egg. When ovulated, the unfertilised rabbit egg, in common with most mammalian eggs, is arrested at metaphase II, awaiting the stimulus to complete meiosis which is normally provided by sperm penetration. A nitrous oxide (N_2O) block is effective in arresting somatic cells in mitosis, and the effect is reversible¹⁴⁻¹⁶. This technique was therefore applied to rabbit morulae in these experiments to increase the proportion of nuclei in mitosis in the donor cell population. In 54 morulae (1,191 nuclei) incubated for 4 h in an atmosphere containing N_2O at a pressure of 5 kg cm^{-2} , 202 nuclei (16.9%) were in mitosis, three times as many as in an untreated population.

To identify the donor nucleus, or its cleavage products, after transfer to the egg, tritiated thymidine ($^3\text{H-TdR}$; specific activity $12.6 \text{ Ci mmol}^{-1}$, Radiochemical Centre) was added to the culture medium of the donor embryos to give a final concentration of $0.25 \mu\text{Ci } ^3\text{H-TdR ml}^{-1}$. After 4 h of incubation, 91.6% of non-synchronised, and 87.8% of 'synchronised' nuclei were labelled.

To obtain single cells for use in nuclear transfer experiments the zona pellucida was cracked, the morula cells were released and where necessary cells in clumps were separated by bouncing them apart on an agar mattress.

In all operations to transplant nuclei into eggs, whether by microinjection or by virus-induced fusion, the eggs and cells were manipulated in the well of a glass chamber on a microscope stage at magnification of $\times 200$ – $\times 400$. The

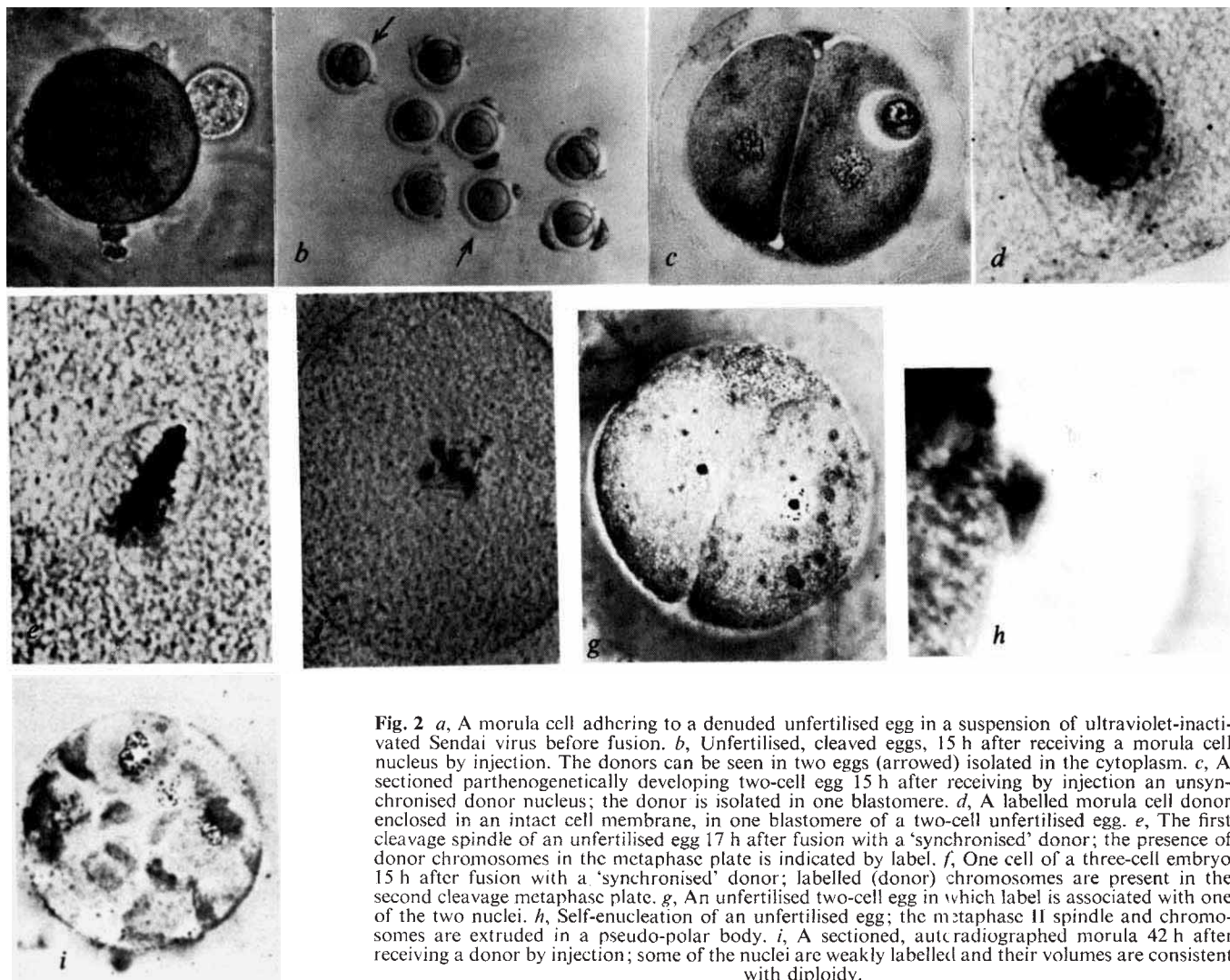


Fig. 2 *a*, A morula cell adhering to a denuded unfertilised egg in a suspension of ultraviolet-inactivated Sendai virus before fusion. *b*, Unfertilised, cleaved eggs, 15 h after receiving a morula cell nucleus by injection. The donors can be seen in two eggs (arrowed) isolated in the cytoplasm. *c*, A sectioned parthenogenetically developing two-cell egg 15 h after receiving by injection an unsynchronised donor nucleus; the donor is isolated in one blastomere. *d*, A labelled morula cell donor enclosed in an intact cell membrane, in one blastomere of a two-cell unfertilised egg. *e*, The first cleavage spindle of an unfertilised egg 17 h after fusion with a 'synchronised' donor; the presence of donor chromosomes in the metaphase plate is indicated by label. *f*, One cell of a three-cell embryo 15 h after fusion with a 'synchronised' donor; labelled (donor) chromosomes are present in the second cleavage metaphase plate. *g*, An unfertilised two-cell egg in which label is associated with one of the two nuclei. *h*, Self-enucleation of an unfertilised egg; the metaphase II spindle and chromosomes are extruded in a pseudo-polar body. *i*, A sectioned, autoradiographed morula 42 h after receiving a donor by injection; some of the nuclei are weakly labelled and their volumes are consistent with diploidy.

Table 1 Introduction of a morula cell nucleus into an unfertilised rabbit egg by microinjection

	No. of eggs injected	Eggs surviving* injection procedure		No. of living eggs surviving incubation for up to 42 h	No. of eggs containing a donor nucleus			
		No.	%		1 Cell	Cleaved	Total	% of eggs injected
Donors not synchronised	528	372	70	271	15	12	27	5.1
Donors 'synchronised'	65	56	86	39	3		3	4.5
Total	593	428	72	310	18	12	30	5.0

*Survival assessed immediately after injection.

chamber was cooled to 5 °C to administer a cold shock to the eggs as a stimulus to activation and to assist adsorption of the virus.

Eggs for injection were held in a cup, a donor cell was taken into a pointed micropipette of 8–10 µm inner diameter and injected into the egg (Fig. 1). In virus-induced fusion experiments the denuded eggs and donor cells were fused by ultraviolet-inactivated Sendai virus at a concentration of 300–400 haemagglutinating units of virus per ml. After exposure of denuded eggs and donor cells separately to the virus suspension for 10 min each egg was rolled on to a cell, which then adhered to the vitelline membrane before fusion (Fig. 2a).

In only 30 of 593 eggs (5%) which received a morula cell nucleus by injection could the donor be identified positively in the egg (Table 1). A higher proportion (18.8%) of nuclei were identified in eggs after introduction by virus-induced fusion (Table 2).

nucleus having been lost by self-enucleation. Of 155 injected eggs examined after nuclear transfer and culture for 13–27 h, 7.1% did not contain an egg nucleus, and in a further 5.2% the egg chromosomes were dispersed in the cytoplasm and so unlikely to take part in development. Of the anucleate eggs, 63.6% (4.5% of all injected eggs) had extruded the egg metaphase II chromosomes in a pseudopolar body (Fig. 2h). Evidence suggesting that nuclear transplantation into enucleated eggs has been achieved is provided by four morulae cultured for 42 h after the injection of a labelled donor. These morulae had cleaved regularly to 18–26 cells, at a rate similar to that of the fertilised egg *in vivo*. Label at a low level was associated with 40 of 86 nuclei (46.5%), and the nuclear volumes were consistent with diploidy (Fig. 2i).

Clearly a somatic cell nucleus, transplanted into an unfertilised rabbit egg, can replace the sperm in supporting development in the early cleavage stages. After 'fertilis-

Table 2 Introduction of a morula cell nucleus into an unfertilised rabbit egg by virus-induced fusion

	No. of eggs	Eggs surviving* fusion procedure		No. of living eggs surviving incubation for up to 27 h	No. of eggs containing a donor nucleus			
		No.	%		1 Cell	Cleaved	Total	% of eggs fused
Donors not synchronised	54	38	70	20	7		7	13
Donors 'synchronised'	47	47	100	38	9	3	12	25.5
Total	101	85	84	58	16	3	19	18.8

*Survival assessed immediately after removal from virus suspension.

After nuclear transfer either by microinjection or by virus-induced fusion, in the best cases the recipient eggs were activated and cleaved regularly, at a rate similar to that of the fertilised egg *in vivo*. In eggs containing both host and transplanted nuclei the egg nucleus usually developed independently of the donor but in some cases the two nuclei fused. Donor nuclei which did not fuse with the egg nuclei were found in one blastomere of eggs that had cleaved (Fig. 2c), where they either became pycnotic, or remained unchanged where isolated from the egg cytoplasm by an intact cell membrane (Fig. 2d). When nuclei fused, a single spindle and metaphase plate formed at the centre of the egg on which both egg (unlabelled) and donor (labelled) chromosomes assembled (Fig. 2e).

Evidence of fusion of egg and donor nuclei has been obtained only in experiments in which 'synchronised' donors have been transplanted into the eggs. An interpretation, consistent with evidence from other studies concerned with somatic cell hybridisation¹⁷, is that for the donor nucleus to combine with the egg nucleus, and then to take part in apparently normal division, both nuclei must be in synchrony. The evidence indicates that nuclei in or near division, that is late G₂, M or G₁, may become synchronised with the egg, whereas nuclei in S phase are asynchronous and do not take part in normal development. It may be supposed that the degree of synchrony necessary to permit fusion of donor and egg nuclei in a normal unfertilised egg is also required for the successful substitution of donor for host nucleus in an egg that has been enucleated.

In some cases the first cleavage metaphase plate may have comprised only labelled (donor) chromosomes, the egg

ation' of the egg by the transplanted nucleus the synkaryon or 'zygote' is capable of apparently normal division at least up to the morula stage. Although such development is rare, the fact that it is possible extends to the rabbit, and by inference to other mammals, the possibility of experiments which have so far been restricted to amphibians. Conclusive proof of the development of an embryo from a somatic cell nucleus transplanted into an enucleated rabbit egg has not been demonstrated; however, there is no apparent reason why a donor nucleus which can fuse with the egg nucleus after transplantation should not also be able to support development by taking the place of the egg nucleus in an anucleate egg.

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Motility, diffusion and cell concentration affect pair formation in *Escherichia coli*

At the high cell densities normally used in *E. coli* matings (about 10^8 cells per ml) individual cells undergo frequent and repeated collisions, and should therefore have multiple opportunities for pairing^{1–3}. A mating pair of cells, once formed, will similarly continue to collide almost as frequently with other cells. It is not known whether these secondary collisions affect either the speed or extent of DNA transfer, since the true properties of a mating pair of cells formed with little likelihood of further collisions have not been studied. Pairs formed at low cell concentrations, however, should be suitable for such studies; indeed, they could be regarded as paradigms of simple cell-cell interactions. We show here that at low densities pair formation is much more efficient than at high densities, and also that motility decreases this efficiency once cells have collided.

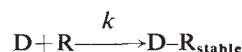
It was necessary to estimate both the number of collisions between cells and the number of pairs formed. Cell movement was observed directly and the formation of progeny was used as an index of pairs formed. Since shaking introduces additional relative cell movement⁴, all matings were carried out without shaking.

In the low density matings, inputs of both donor and recipient cells of about 10^5 per ml gave well defined progeny counts and showed exponential growth of both parents during 1 h. The selected marker, *purE*⁺, enters the recipient cell approximately 4 min after DNA transfer starts⁵. The formation of progeny and the viable counts during such an experiment using non-motile strains are shown in Fig. 1. The rate of formation of progeny accelerates steadily, and their number does not exceed 0.1% of either parent during the experiment. The lag in the appearance of progeny strongly suggests that an unstable pairing which is sensitive to the experimental handling conditions precedes the more stable pairing which survives the pipetting and mild vortexing used. This suggests the model



where D represents donors, R recipients and $D-R_{\text{stable}}$ the complexes which give rise to progeny colonies. For practical

purposes, however, we have estimated a minimum value for the rate constant from the simplified model



assuming a time lag for the conversion of any pair into a stable pair of about 9 min, as the data suggest.

The experimental data from low density matings could be fitted to the simplified model at early times but the numbers of progeny found during the later stages of the experiment consistently exceeded expectation. We have observed under the microscope that both cells in apparent mating pairs, when placed on agar blocks and incubated further, continue to divide. We have therefore assumed that the number of progeny cells (*P*) can increase due to multiplication at a rate characteristic of the recipient strain. The equations used to model the process were

$$\begin{aligned} dP/dT &= kDR + k_R P \\ dD/dT &= -k_D D \\ dR/dT &= -k_R R \end{aligned}$$

where k_D and k_R are the observed growth rate constants for donor and recipient respectively. The theoretical numbers of progeny were computed using the cell numbers at the time of mixing as the initial conditions. The value of the second order rate constant, *k*, used to generate the theoretical curve (which incorporates the lag previously mentioned) shown in Fig. 1 was 7×10^8 l per 6×10^{23} cells per s. These units facilitate comparison of the rate constant with the upper limit for a second order rate constant in liquids for a reaction between neutral molecules, 3×10^9 l mol⁻¹ s⁻¹, in a diffusion-limited reaction⁶. Pairing is therefore an inherently efficient process following the initial contact, as suggested by Nelson¹⁰, particularly since not all cells in the population may be competent to mate^{4,10} and some mating pairs will fail to produce recombinants for the selected marker¹¹.

The apparent rate constant for the mating (at low density) between motile parental strains is, as expected, significantly greater than for non-motile strains (Table 1). The apparent rate constants for the two crosses involving one motile and one non-motile parent, which might be expected to be similar, show an interesting asymmetry; the cross between a motile donor strain and a non-motile recipient strain is more fertile than the complementary cross. But the significance of the difference between the first two crosses mentioned, and of the asymmetry in the latter two crosses, can be evaluated only if the contribution of cell movement to the collision rate is known.

To this end, we included in these experiments measurements of cell movement. During each mating, videotape records were made of microscopic fields of samples from the parental cultures, observed in a closed chamber at 37 °C, while the bulk cultures were mated. The recordings were replayed to obtain

Table 1 Effect of motility and concentration on the relative efficiency of mating pair formation

Mean parental cell numbers per ml	Motility of strains Donor Recipient	Relative mutual diffusion coefficients	Observed rate constant $\times 10^{-10}$ (l per 6×10^{23} cells per s)	Relative efficiency of pair formation
1.6×10^5	— —	1	0.07	1
	+ +	19.8	0.6	0.43
	+ —	9.3	0.6	0.92
	— +	11.4	0.09	0.11
3.2×10^6	— —	1	0.1	1.43
	+ +	48.8	1.3	0.38
3.6×10^8	— —	0.8	0.006	0.11
	+ +	4.0	0.0015	0.0054

Three sets of matings were carried out; within each set there was an approximate 1:1 ratio of donor to recipient cells, and a similar total cell input. The donor strains were B8 (motile) and ED1064 (non-motile); recipient strains were X478 (motile) and ED1065 (non-motile). The values of the relative mutual diffusion coefficients and of the relative efficiencies of pair formation were chosen to be 1 for the cross between non-motile parental strains at the lowest concentration.

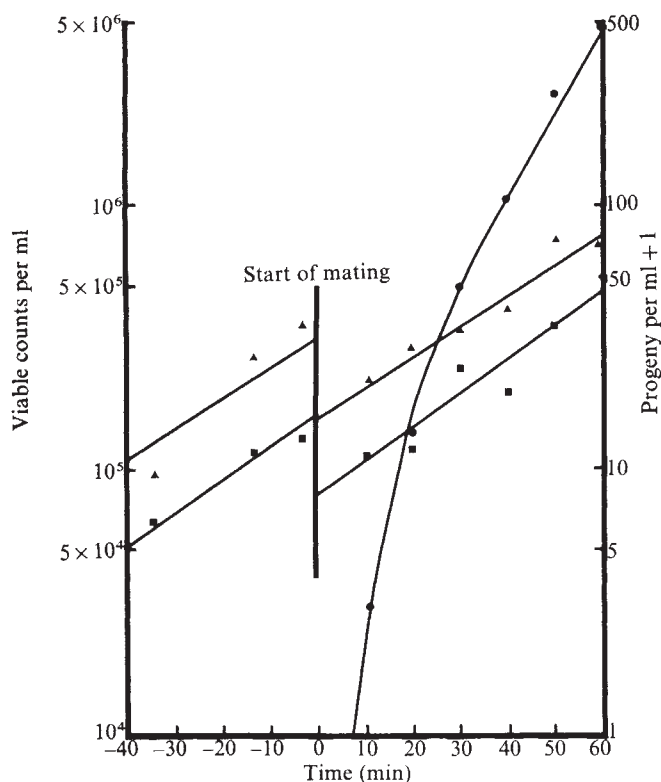


Fig. 1 Progress of the mating between the *E. coli* strains ED1064 and ED1065. These were isolated as spontaneously arising non-motile derivatives from, respectively, cells of Hfr B8 (refs 5 and 6; *metB*) and χ 478 (ref. 7; *F⁻proC purE trp lys metE leu lac str*) that had survived a challenge from bacteriophage χ (provided by E. Meynell), which only attacks motile cells⁸. Cultures were grown in L broth at 37 °C to about 2×10^8 cells per ml, diluted to about 10^4 cells per ml in fresh L broth and grown for a further 90 min to approximately 2×10^8 cells per ml in a rotary shaking water bath (100 r.p.m.). Equal volumes of donor and recipient cultures were then mixed; incubation continued without shaking. Separate samples were diluted as appropriate in buffer, vortexed gently and pipetted into molten agar held at 46 °C; these samples were then poured on to different supplemented minimal agars selective for *Pur⁺* (*Str^r*) progeny and also for each parental strain. Viable counts per ml of donor (Δ) and recipient ($\blacksquare$) and the number of progeny per ml + 1 ($\bullet$) are shown semi-logarithmically with the curve of the predicted progeny per ml + 1, assuming that $k = 7 \times 10^8$ l per 6×10^{23} cells per s.

tracings of the tracks of individual cells for the calculation of apparent diffusion coefficients. The relative magnitudes of the upper limits for the rate constants are directly proportional to the sum of the apparent diffusion coefficients for donor and recipients; we assume that the cell-cell distance on contact is the same in all crosses. From the ratio of the observed rate constants to the relative upper limits in these matings, we can calculate the relative efficiencies of pair formation per collision (Table 1).

The most efficient matings are those between the two non-motile strains at the lower densities. The corresponding crosses between the motile strains B8 and χ 478 are about one-third as efficient. Since the average interval between successive collisions involving a given cell at the lowest concentration exceeds the duration of the experiment, this lower efficiency is an inherent property of mating pairs of motile cells.

The relative efficiencies of the matings involving one motile and one non-motile parent show that the asymmetry noted above is not due to different mobilities of strains B8 and χ 478. Therefore other possibilities, including vectorial movement by the donor strain, must be considered.

Cell concentration also affected the relative efficiency. Thus, when one allows for a tenfold drop in the diffusion coefficient of the motile cells (but not of the non-motile cells) at the

highest concentration, the crosses between motile cells and between non-motile cells were both ten times less efficient. These low efficiencies may be due to the additional collisions, which could result in disruption of existing pairs or the addition of further cells to existing complexes. We therefore suggest that physiological and genetic analysis of low and moderate density matings will be the most reliable source of information about the behaviour and properties of a simple mating pair of cells.

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Migration and chemotaxis of anucleate cytoplasmic leukocyte fragments

KNOWLEDGE of the mechanisms involved in leukocyte locomotion is limited. Although it is known that amoebae require a nucleus for locomotion^{1,2} no similar studies have been done with leukocytes. Nothing precise is known about the cellular factors involved in the perception of a chemotactic gradient and the translation of this information into directional migration. We have investigated this problem using anucleate cytoplasmic fragments and heat-polarised granulocytes.

When leukocytes were heated to 46 °C on a microscopic stage under a coverslip, pronounced pseudopods were produced, resulting in anucleated cytoplasmic fragments. The stages of this process are summarised schematically in Fig. 1. Heating resulted in the gradual polarisation of the cell into two parts (Fig. 1a–d), one containing the nucleus and most of the phase-dense granules, the other consisting mainly of the hyaloplasm, containing few or no phase-dense granules. When polarisation occurred the cytoplasmic part moved away from the less mobile nucleated section. This process was reversible if heating stopped. Continued heating, however, resulted in the irreversible formation of anucleated cytoplasmic fragments (Fig. 1e). The behaviour of cells and fragments was analysed by microcinematography and video recording and, subsequently, the cell path was traced.

We consistently observed random migration of viable cytoplasmic fragments, while the nucleated part showed little or no motility. Previous studies had shown that neutrophils respond chemotactically to red blood cells which have been destroyed by a laser microbeam (necrotaxis)⁴. We found that viable cytoplasmic fragments are also capable of directional migration, resulting in the accumulation of fragments around the chemotactic target. We have demonstrated that the fragments can ingest bacteria³. Thus in contrast to amoebae^{1,2}, granulocytes do not require a nucleus for locomotion and chemotaxis, at least not for a limited time. Furthermore, only part of the cell membrane is needed for recognition of a chemotactic gradient and for phagocytosis. This conclusion agrees with observations⁵ showing a segmental response of macrophages to phagocytic stimuli. It is interesting to compare the

functional capacity of cytoplasmic fragments from leukocytes with the behaviour of blood platelets, which also represent cytoplasmic fragments. Even though it is well established that platelets contain contractile elements⁶, we could not detect locomotion or chemotaxis. The structural basis for the differences between leukocyte fragments and platelets remains to be studied.

We have analysed the behaviour of heat-polarised leukocytes in order to obtain further information about the mechanism of the chemotactic response. In the absence of chemotactic stimulation, heat-induced pseudopod formation occurs randomly in all directions. Chemotactic stimulation considerably enhances heat-induced pseudopod formation, particularly in the early phases (Fig. 1*b* and *c*). More than 90% of the pseudopods extended and moved towards the chemotactic target, while the nucleated part either remained in its original position or slowly followed the cytoplasmic part. It has been suggested that leukocytes recognise a chemotactic gradient by means of receptors on the cell surface^{7,8} and that pseudopod formation, leading to directional migration, takes place at the site of the membrane, which undergoes the most intense chemotactic stimulation⁹. This was evaluated further by studying the behaviour of heat-polarised cells, orientated in such a way that the nucleated part was nearest to the dying red cell and the cytoplasmic pseudopod extended away from the chemotactic target (Fig. 2, 1). Various polarised cells differed in their response to chemotactic stimulation, as shown by the following examples. Occasionally a cell withdrew the distant pseudopod into the nucleated part and extended a new pseudopod towards the chemotactic red cell, as expected. More cells, however, behaved differently. The distant pseudopod migrated around the nucleated part towards the red cell, causing the nucleated section to be dragged away from the red cell, thus rotating the entire cell by 180° (Fig. 2*a*). Another cell showed a mixed response (Fig. 2*b*). Chemotactic stimulation resulted in the formation of a leading edge (metapod) on the nucleated part nearest to the destroyed red cell, as well as migration of the distant pseudopod around the nucleated part. At an early stage both sections of the cell seemed to migrate independently towards the chemotactic target. This independence of action by two parts of the cell indicated that different sections of the cell can recognise and respond separately to chemotactic stimulation. Later the more actively migrating pseudopod directed the entire cell towards the red cell, dragging the nucleated part behind it.

Fig. 1 Stages of fragment formation.

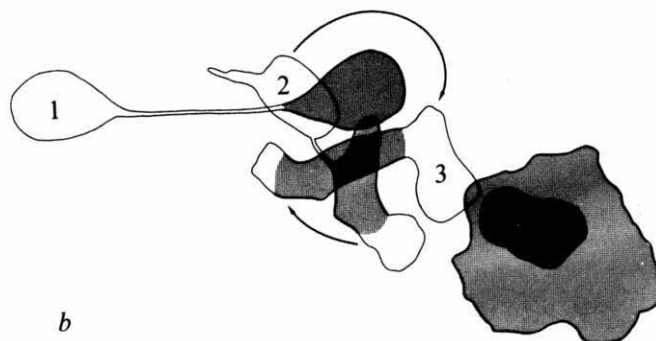
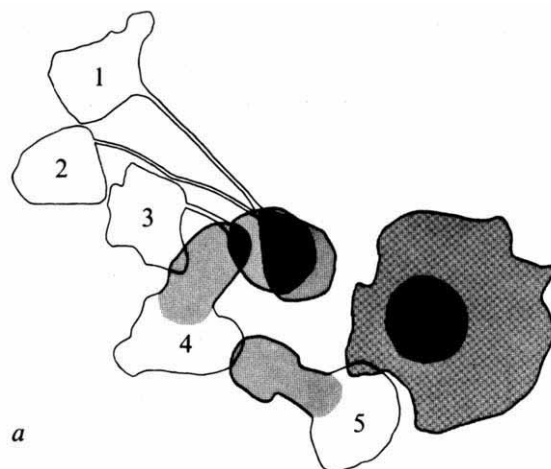
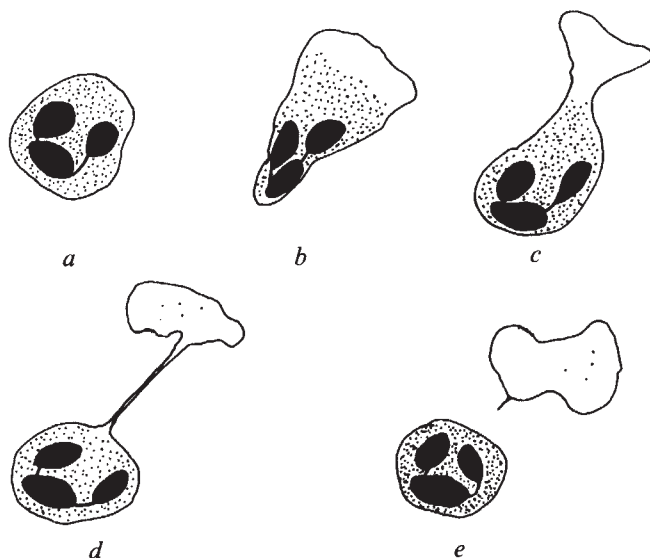


Fig. 2 Examples of polarised leukocytes responding to chemotactic stimulation: tracings of the path. Position of the leukocyte before chemotactic stimulation (1) and subsequent positions after chemotactic stimulation (2-5); nucleated part of the leukocyte (solid grey), position of intact (dark shading) and lysed red cells (light shading).

Analysis of the chemotactic behaviour of heat-polarised cells reveals that these cells have different means of reorientation. There is, however, one common feature—the rearrangement of the hyaloplasm to form a leading edge (metapod). In these experiments (Fig. 2) the nucleated part often did not respond or responded to a limited extent, although this section of the cell underwent intense chemotactic stimulation. The antagonistic effect of the segmental responses observed in the cell shown in Fig. 2*b* suggests that migration is not necessarily coordinated by a single pacemaker per cell.

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Release of eosinophil chemotactic factor from human polymorphonuclear neutrophils by calcium ionophore A23187 and phagocytosis

THE number of mediators assumed to be important in the pathogenesis of acute allergic reactions has increased considerably recently. At the outset, Dale¹ suggested that histamine was the critical feature of such responses; then a role for the slow reacting substance of anaphylaxis (SRS-A) was suggested by Brocklehurst², one for the eosinophil chemotactic factor of anaphylaxis (ECF-A) by Kay and Austen^{3,4} and more recently a kallikrein-like molecule has been described⁵. Whether preformed, like histamine, or synthesised after the immune event, like SRS-A, each of these mediators had two features in common. (1) It could be released (in primates) only after an IgE antibody-antigen interaction and (2) the cell of origin was the mast cell or the basophil. These generalisations can no longer be accepted. We have described the generation and release of SRS-A from human polymorphonuclear neutrophils (PMN) by the calcium ionophore A23187 (ref. 6), and we report here ionophore-induced release of eosinophil chemotactic

release of ECF from whole peripheral leukocyte preparations (obtained by dextran sedimentation¹⁵), the amount of activity obtained far exceeded that released with antigen, whereas histamine release observed was similar with both stimuli. When human basophil-mononuclear cell preparations, obtained by Ficoll-Hypaque flotation¹⁶, were challenged with the ionophore, the release of the two mediators was similar to that obtained with antigen or anti-IgE stimulation. With purified PMN cell preparations¹⁶, however, ionophore stimulated the release of more ECF than could be accounted for by basophil contamination. The results shown in Table 1 are representative of eight similar experiments. No lactate dehydrogenase (LDH) was released from the PMN exposed to ionophore.

The PMN-derived ECF preferentially attracts eosinophils, whether from human or guinea pig sources. Control experiments demonstrated that the concentrations of ionophore used experimentally did not cause chemotaxis, nor did the ionophore generate further chemotactic activity in ECF containing cell-free supernatants. Chromatographic analysis of the PMN-derived ECF on Sephadex G-25 demonstrated a single peak of eosinophil chemotactic activity with a molecular weight of approximately 600, identical to that found in chromatographic analysis of antigen or anti-IgE-induced ECF-A from purified basophil preparations.

Having established that ECF could be produced by human

Table 1 Cell separation study of human leukocytes

	Lymphocyte preparation ECF (Eos. per HPF)	Histamine ($\mu\text{g ml}^{-1}$)	PMN preparation ECF (Eos. per HPF)	Histamine ($\mu\text{g ml}^{-1}$)
Buffer control	3	0.04	7	0.01
Ionophore ($5\mu\text{g ml}^{-1}$)	190	0.45	1,200	0.06
a-IgE ($3 \times 10^{-2} \mu\text{g ml}^{-1}$)	150	0.38	14	0.06

HPF, high power field; Eos, eosinophils.

This table shows mediator release from 2.5×10^7 cells treated with calcium ionophore A23187 and anti-IgE. Histamine release in the PMN preparation was very low, and so was release by reverse anaphylaxis with anti-IgE. Ionophore-induced ECF release from PMNs, however, was massive. Precision of the chemotaxis assay was 15.8%. Differentials were:

	PMN	Eosinophils	Basophils	Lymphocyte + Monocyte
Lymphocyte preparation (%)	0.0	0.0	6.2	93.8
PMN preparation (%)	95.1	1.5	0.9	2.6

factor (ECF) from that cell type. More importantly, we have been able to demonstrate that this mediator can be released by normal human PMN during phagocytosis. (We prefer to call the leukocyte-derived factor described here and in our previous publications ECF for two reasons: first, ECF is not associated only with anaphylaxis, and second, complete biochemical identity with the previously described ECF-A has not been proved.)

ECF-A is a preformed, low molecular weight peptide which can be released from various mast cell or basophil sources^{3,4,7-9}. It is also released from human peripheral leukocytes by reversed anaphylaxis (anti-IgE), or with antigen exposure in the case of sensitised cells¹⁰. Our assay for ECF has been described before¹⁰. Briefly, 2.5×10^8 cells containing at least 25% human or guinea pig eosinophils were placed above a polycarbonate filter in a modified Boyden chamber, and the substance to be tested for chemotactic activity placed below the filter. After 3 h of incubation at 37 °C, the cells that have migrated completely through the filters were counted under the microscope at $\times 100$ magnification. Antigen dose response and kinetic relationships for ECF release were identical to those for the release of histamine.

The line of investigation reported here is derived from studies with the calcium ionophore A23187, which has been shown to stimulate various secretory reactions^{11,12} and to be a potent histamine-releasing agent in mast cells¹³ and basophils¹⁴. When the ionophore was used to study the

PMN, we investigated if it could be released by physiological stimuli. Since phagocytosis leads to the secretion of a number of enzymes^{17,18}, we studied this phenomenon for the release of ECF from cells. As a phagocytic stimulus, we used a standard preparation of zymosan particles treated with normal human serum, (Z(x)), that is, coated with complement factors and then washed extensively. The zymosan particles and human PMN were incubated together at 37 °C and the supernatants were removed by centrifugation at different times and checked for ECF

Table 2 Kinetic studies of ECF generation

Time (min)	βG (ΔA)	ECF (Eos. per 5HPF)	LDH	NBT (ΔA)
10	0.052	1,325	Baseline	0.110
30	0.070	1,550	Baseline	0.130
90	0.070	200	Baseline	0.130

Z(x) (1.5 mg) was incubated with 2.5×10^6 PMN in a final volume of 1 ml. After various times the mixture was centrifuged, and the supernatants assayed for chemotactic activity. For β -glucuronidase (βG) determinations, 100 μl per ml of the supernatant was incubated with 0.01 M phenolphthalein glucuronic acid and 0.5 ml of 0.1 M acetate buffer, pH 5.0, for 12 h. Absorbance (A) was read at 540 nm for βG and at 360 nm for LDH. (ΔA represents the reading at each time, minus cell and buffer control.) For the NBT test, extracted formazan was read at 515 nm. Supernatants of Z(x) alone did not show chemotactic activity. Values obtained were comparable in four different experiments.

activity. β -Glucuronidase and LDH release were measured by standard techniques¹⁹, as was the nitroblue tetrazolium (NBT) reduction of the cells during phagocytosis²⁰.

As Table 2 shows, release of the lysosomal enzyme marker β -glucuronidase paralleled phagocytosis as indicated by NBT reduction; cell viability was maintained since there was no release of the cytoplasmic enzyme marker LDH. Phagocytosis also led to highly significant ECF release, which at first paralleled the release of β -glucuronidase, but by 90 min, the ECF activity in the supernatant was markedly decreased.

The chemotactic factor released by phagocytosis was as highly selective for guinea pig and human eosinophils as that derived by ionophore stimulation. Sephadex G-25 chromatography revealed an apparent molecular weight of 600, similar to the ECF preparations described here. The ECF therefore seems to be distinct from the non-dialysable chemotactic factor described by Zigmond and Hirsch²¹ generated from human PMN during phagocytosis of aggregated gamma globulin. This latter factor also differs in that, unlike ECF, it attracts neutrophils as well as eosinophils.

Kinetic studies of ECF generation (Table 2) during phagocytosis indicated that, after reaching a peak, the chemotactic activity decreased rapidly. Results of preliminary experiments suggest that this decrease results from the destruction of ECF. Sonication of purified PMN led to little or no eosinophil chemotactic activity, but when sonicates were mixed with supernatants obtained from phagocytosing cells, there was a distinct reduction of chemotactic activity. The nature of the material(s) which caused destruction or inactivation of ECF in sonicates and during the late phase of phagocytosis is under study.

The data reported here indicate that ECF is present in human PMN and that it is released during phagocytosis. This finding is novel in that this mediator of the allergic response was thought to exist only in mast cells and basophils and to be released only after IgE-antigen interaction. Because PMN are present in the human body in much greater numbers than either basophils or mast cells, the quantitative contribution of these cells to inflammatory events is likely to be of great importance. These rather surprising observations further blur the artificially drawn lines between the various types of inflammatory processes. If the same mediators can be released by IgE-mediated processes from basophils, or from PMN by phagocytosis, the differentiation between these processes becomes more subtle and complex.

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Role of stem cell migration in initiation of mouse foetal liver haemopoiesis

THEORIES of the development of mammalian hepatic haemopoiesis have polarised around two viewpoints. One hypothesis suggests that multipotential haemopoietic stem cells (HSC) migrate from the yolk sac and colonise the developing hepatic primordia¹. According to the other view, hepatic haemopoietic tissue arises from the transformation of liver mesenchyme cells and thus has no direct relationship with vitelline haemopoiesis². To solve this developmental problem, it is necessary to establish techniques, either *in vivo* or *in vitro*, to maintain hepatic tissue in a foetal state. *In vitro* studies have demonstrated that hepatic haemopoietic tissue can develop if tissues are explanted after the 28-somite stage³. Thus, these experiments did not verify the inductive model² of hepatic haemopoiesis, although the *in vitro* conditions may have curtailed haemopoietic induction without altering hepatic differentiation. With an exogenous source of yolk-sac-derived HSC attempts have been made to colonise pre-28-somite hepatic explants *in vitro*, using the appearance of granulopoiesis as an indication of successful recombination. These experiments were not conclusive since granulopoiesis

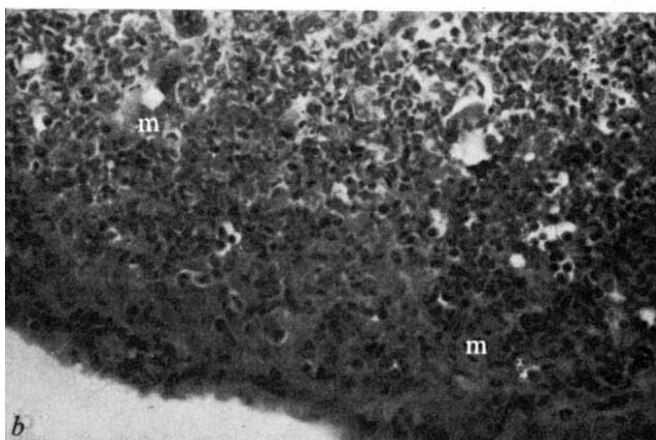
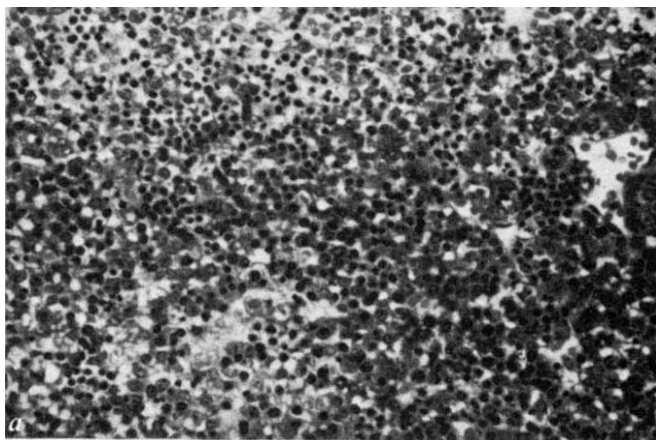


Fig. 1 a, Section of 15-d CBA foetal liver, erythroid differentiation predominates ($\times 168$). b, Section of 13-d CBA foetal liver, sectioned after being grafted on to kidney for 2 d. Granulocytes in various stages of differentiation are apparent. Mitotic figures (m) can also be observed ($\times 168$).

was observed with yolk sac cells *in vitro* in the absence of hepatic tissue (G.R.J., unpublished).

To overcome some of these problems we have used an *in vivo* grafting technique. Hosts were prepared in various ways, either being non-irradiated, irradiated (850 r.) or partially hepatectomised; they included BALB/c *nu/nu* (nude), (CBA $\times$ C57BL) S1/S1^d, CBA and (CBA $\times$ C57BL) F₁ mice. Tissues for grafting were obtained from foetuses of the CBA or (CBA $\times$ C57BL) F₁ strains. Females of known gestational age (day of vaginal plug discovery equals day zero of gestation) were killed by cervical dislocation and foetal hepatic tissue was removed aseptically as before³. Intact hepatic tissue, obtained from foetuses younger than 11.5 d of gestation (36 somites), was placed under the kidney capsule of syngeneic 8-week-old mice. With older hepatic tissue, portions not larger than 2 mm³ were placed on to a graft bed prepared by shaving off a thin slice of host renal cortex⁴. One graft per host was our standard procedure. After suitable intervals, hosts were killed and the kidneys with attached grafts were removed, fixed in Bouin's fixative, sectioned and stained with haematoxylin and eosin.

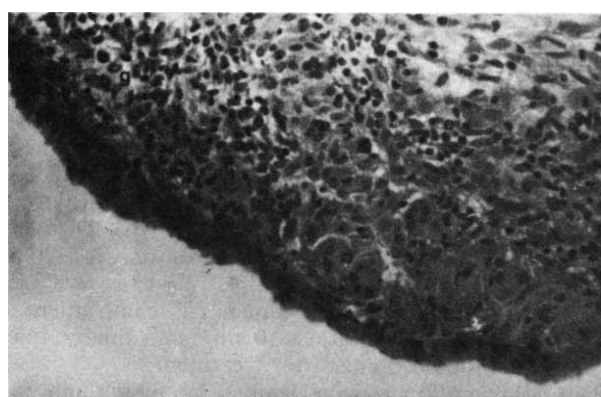


Fig. 2 Section of 24-somite CBA foetal liver graft after 7 d under the kidney capsule of a S1/S1^d host. g, Granulocytes; e, erythroblasts ($\times 168$).

With all grafts that were initially older than 28 somites, haemopoiesis declined rapidly and 10 d after grafting only some connective tissue remained, surrounded by extensive necrotic areas. Cell suspensions derived from the grafts were counted and subjected to differential cell counts. *In vivo* spleen colony assay for HSC and the *in vitro* agar assay for granulocyte-macrophage progenitors (CFC) were performed as before¹. All these parameters verified the haemopoietic decline, HSC and CFC were practically absent by 6 d and at the same stage the cellularity had been reduced by 90%. In all host combinations a marked morphological change was observed in the graft tissue, the predominately erythroid tissue⁵ becoming granulocytic (Fig. 1). This subjective view was verified by differential cell counts on smears derived from graft cell suspensions; up to 44% of the haemopoietic cells within 2 d of being grafted were of the granulocyte lineage (Table 1).

Table 1 Percentage figures for erythroblasts and granulocytes from normal 13-d and 15-d CBA foetal livers and of 13-d CBA foetal liver grafted on to host kidney cortex for 2 d (15 d equivalent).

	Erythroblasts (%)	Granulocytes (%)
13-d gestation CBA foetal liver	88	8.25
15-d gestation CBA foetal liver	92.75	5.5
13-d gestation CBA liver, 2 d in irradiated F ₁ host	20.5	44.6
13-d gestation CBA liver, 2 d in non-irradiated F ₁ host	41.25	30.75

Figures obtained from smears, 500 haemopoietic cells scored, numbers of erythroblasts and granulocytes are expressed as a percentage of the total number of haemopoietic cells.

Numerous mitotic figures were also observed within the masses of granulocytes (Fig. 1).

With hepatic grafts younger than the 28-somite stage, some hepatic differentiation appeared to continue, even when sampled 10 and 12 d after grafting. Morphological integrity was maintained in these grafts and by 10 d duct systems had developed, necrotic areas being virtually absent. At no stage, however, was haemopoietic tissue observed in pre-haemopoietic grafts that had been placed in non-irradiated, irradiated, nude or partially hepatectomised hosts. With S1/S1^d hosts, however, small areas of erythroid and granuloid tissue were observed when grafts were sampled after a minimum period of 7 d (Fig. 2). In this situation grafts as early as the 22-somite stage displayed some haemopoietic areas.

These results may be interpreted as indicating that an exogenous supply of haemopoietic cells is necessary for the establishment of haemopoietic tissue in the developing foetal liver. When no haemopoietic tissue developed, the host either had no HSC (irradiated hosts) and therefore could not supply the graft with cells, or had normal haemopoietic tissue (non-irradiated, partially hepatectomised or nude hosts) and therefore presumably no requirement for extra-haemopoietic areas. In contrast S1/S1^d mice are anaemic but when transplanted with exogenous haemopoietic tissue, they support haemopoiesis. Further substantiation for the need of an exogenous source of haemopoietic cells for the initiation of hepatic haemopoiesis was gained from the following experiments. Hosts bearing post-28-somite hepatic grafts were irradiated (850 r.) and then reconstituted with 10⁷ syngeneic bone marrow cells. Seven days after irradiation-reconstitution grafts displayed prominent erythroid, megakaryocytic and granuloid colonies, the latter being the more frequent (Fig. 3). Control grafts placed in hosts subsequently irradiated but not grafted with bone marrow did not reveal haemopoietic colony formation.

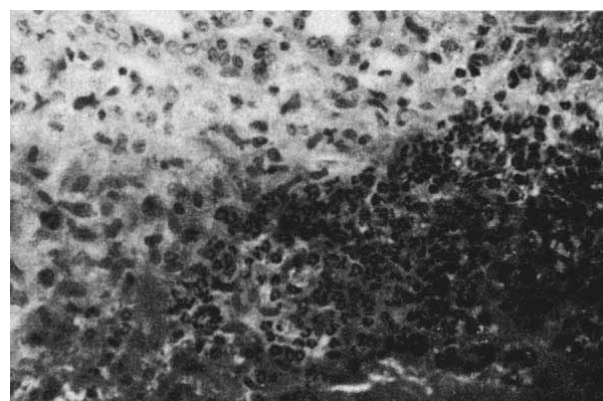


Fig. 3 Granulocyte colony in graft of 16-d CBA foetal liver, irradiated after grafting, and reconstituted with syngeneic bone marrow cells on day of grafting. Section sampled 7 d after grafting ($\times 168$).

Although haemopoiesis did not persist in hepatic grafts, observations made during these experiments are interesting. The mouse foetal liver is predominately erythroid with up to 90% of the haemopoietic cells in 13-d gestation animals differentiating along this pathway⁵. Since the incidence of CFC in foetal hepatic tissue is as high or higher than in adult marrow⁶ the lack of extensive granulopoiesis suggests that either microenvironmental or external regulatory factors suppress this line of differentiation. In our experiments foetal hepatic tissues were grafted into adult hosts and any external foetal inhibitory factors may have been bypassed, thus allowing granulocyte expression. This removal of inhibition may also be reinforced by colony stimulating factor (CSF) present in normal mouse sera⁷. Therefore, with elevated levels of this substance a further increase in granulopoiesis would be expected. Such an increase of CSF has been reported in the serum of irradiated mice⁸ and in our experiments grafts placed into irradiated hosts

contained higher percentages of granulocytes than grafts derived from non-irradiated hosts (Table 1).

Supplementing the above proposed humoral affects, alterations in the types of haemopoietic inductive microenvironments⁹ in the grafts could produce the observed results. Thus the increased granulopoiesis may be accounted for by a loss of the erythroid-inducing microenvironment and a stabilisation or increase in the numbers of granulocyte inductive microenvironments.

The fact that pre-haemopoietic foetal liver can support haemopoiesis if supplied with an exogenous source of haemopoietic cells supports the cell-seeding hypothesis¹. The absence of any visible haemopoietic tissue in pre-haemopoietic hepatic grafts, placed into irradiated hosts, seems to invalidate the theory of induction². It is possible, however, that the experimental procedure prevents the inductive event or alternatively that the pre-haemopoietic grafts rapidly lose the factors necessary for the maintenance and subsequent differentiation of HSC. This latter criticism is opposed by the observation that hepatic grafts, which were irradiated after placement on the host kidney, could still produce discrete colonies even if the host was reconstituted 3 d after irradiation. The results reported here therefore suggest that an exogenous supply of HSC is necessary for the development of hepatic haemopoietic tissue. After the attachment of these cells, local microenvironmental and/or external humoral factors then determine their subsequent differentiation.

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Cooperation across the histocompatibility barrier

STUDIES in mechanisms of T-B collaboration in the immune response have shown that T cells can produce factors after stimulation with antigen^{1–3}, allogeneic cells^{4–6}, or mitogens (ref. 7 and O. Sjöberg, unpublished), that can stimulate B cell responses to antigens. Such factors have been categorised as specific^{1,3} or nonspecific (refs 4–7 and O. Sjöberg, unpublished). Studies have shown that the specific factor produced by (T,G–A–L) stimulation of educated T cells⁸ is able to promote antibody formation by both syngeneic and allogeneic cells. Similarly, with one exception⁶, nonspecific factors from antigen- or mitogen-activated T cells were able to cooperate with B cells from histoincompatible strains^{7,9}. Reports that histoincompatible T and B cells could not be demonstrated to cooperate in a humoral immune response¹⁰, in conditions in which it was considered that inhibitory allogeneic effects were excluded or irrelevant, indicated that for efficient collaboration T and B cells need to share surface determinants coded for by major histocompatibility complex (MHC), in particular the I region or its equivalent. One report¹¹ argued against this;

allophenic mice (C3H+C57BL) were able to make non-responder allotype towards (T,G)–A–L. This finding made the presumption that it was the high responder T cells which were collaborating with the low responder B cells, but could not exclude that alternative cooperative mechanisms were operational.

The present studies represent a direct analysis using T cells derived from bone marrow chimaeras¹² and testing them with histocompatible B cells. In this situation, as in allophenic mice, the T cells are rendered specifically unresponsive to the determinants of the H-2 complex of the B cells used in testing for collaboration.

B cells were obtained from anti- θ treated spleens of CBA (H-2^k), BALB/c (H-2^d) or C3H×BALB/c (H-2^k×H-2^d) F₁ mice which had been primed at least 3 weeks previously with 500 μ g trinitrophenyl-fowl gamma globulin (TNP-FGG) in Freund's complete adjuvant (FCA). Helper T cells were derived from the spleens of mice primed with 500 μ g KLH in FCA 3 weeks, and again with 50 μ g soluble keyhole limpet haemocyanin (KLH) 3 d before collection. B cells were in large part removed by passage through nylon wool, and red cells were lysed by treatment with 0.17 M ammonium chloride. Bone marrow chimaeras (C3H+BALB/c) (H-2^k+H-2^d) were derived by transfer of anti- θ -treated bone marrow cells from C3H and BALB/c donors to irradiated F₁ recipients. Mice were primed with KLH to generate helper cells at least 3 months after bone marrow transfer.

Anti-H-2^k (B.10 anti-A/J) and anti-H-2^d (B.10 Br anti-DBA/2) sera were absorbed with either BALB/c or C3H lymphocytes to render them specific for only one type of cell in the chimaera. Where helper T cells were treated with these alloantisera and guinea pig complement they were initially suspended at 2×10^7 ml⁻¹ and finally adjusted to the same initial volume after treatment.

T-B collaboration was determined by mixing appropriate combinations of KLH-primed T cells with anti- θ -treated TNP-FGG-primed B cells in 10 μ l volume in the wells of Terasaki plates¹³ in the presence of 2–5 μ g ml⁻¹ TNP-KLH. After 5 d incubation at 37 °C, 30 wells from each treatment group were sampled for anti-TNP plaque-forming cells (PFCs) by conventional plaquing techniques using TNP coupled to Donkey erythrocytes. Results are displayed as the number of responding cultures and the total anti-TNP PFCs derived from 30 wells.

Table 1 shows results of an experiment which confirms the finding¹⁰ that non-tolerant histoincompatible T and B cells will not cooperate *in vitro*. CBA (H-2^k) T cells

Table 1 Total (direct and indirect) PFC responses (1.5×10^6 per well) of anti- θ -treated TNP-FGG primed spleen cells to 2 μ g ml⁻¹ TNP-KLH in presence of histocompatible and histoincompatible KLH-primed T cells

B cell source	T cells source (No. per well)	No. of positive wells (30 wells assayed)	Total PFCs (direct + indirect PFCs, 30 wells)
BALB/c	BALB/c		
	9×10^5	8	100
	4.5×10^5	13	230
	CBA		
CBA	9×10^5	2	22
	4.5×10^5	2	17
	BALB/c		
	9×10^5	3	22
CBA	4.5×10^5	1	7
	CBA		
	9×10^5	28	871
	4.5×10^5	29	795

Table 2 Ability of histoincompatible T cells (H-2^d) derived from a chimaera to cooperate with H-2^k B cells

B cells	T cells	Antiserum treatment	No. of positive wells (30 wells assayed)	Total anti-TNP-PFC in 30 wells	% Dead T cells
C3H × BALB/c F ₁ (H-2 ^k × H-2 ^d)	F ₁ (H-2 ^k × H-2 ^d)	None	30	8,300	12
	Chimaera (H-2 ^k + H-2 ^d)	None	30	4,245	15
	Chimaera	Anti-H-2 ^k	30	6,484	57
	No T cells	—	0	—	—
CBA(H-2 ^k)	F ₁	None	30	3,469	12
	Chimaera	None	30	2,939	15
	No T cells	—	1	12	—
	F ₁	Anti-H-2 ^k	2	31	94
	Chimaera	Anti-H-2 ^k	30	3,796	57

B cells were from either anti-θ-treated C3H × BALB/c or CNA TNP-FGG-primed spleen cells. T cells were derived from nylon wool-enriched KLH-primed spleen cells. Anti-H-2^k treatment of T cells was carried out by incubating 2 × 10⁷ cells with 1 ml 1:5 dilution of anti-H-2^k antiserum for 30 min on ice, and subsequently with 2.5 ml 1:6 GPC for 30 min at 37 °C. T cells were then spun through foetal calf serum and resuspended to 1 ml. All cultures were challenged with 5 µg ml⁻¹ TNP-KLH, and anti-TNP responses determined after 5 d.

cooperate efficiently with CBA B cells but not with BALB/c cells. Similarly, it is clear that BALB/c T cells (H-2^d) cooperate best with BALB/c cells, and poorly with CBA B cells. We have found it persistently difficult, however, to achieve good responses with BALB/c cells in this system.

This inability of H-2^d cells to function with H-2^k B cells is not seen if H-2^d T cells are derived from an (H-2^k + H-2^d/BALB/c + C3H) chimaera. Table 2 shows the results of an experiment in which anti-H-2^k alloantiserum-treated chimaeric cells cooperate at least as efficiently as untreated T cells with either of (H-2^k × H-2^d) or H-2^k B cells. The efficacy of the anti-H-2^k treatment is clearly demonstrated by the fact that F₁ T helper cell function was eliminated by

alloantigens of the B cell donor will also collaborate efficiently in an *in vitro* primary response of rat spleen cells. Similar results have also been obtained by negative selection procedures in the mouse (J. Quintans, unpublished). It is relevant that our data deal with the responses to TNP-KLH; unlike SRBCs the soluble form of this antigen cannot be helped by allogeneic effects or non-specific factors¹⁶ which cannot be totally excluded in the data of Heber-Katz and Wilson. The original experiments of Katz *et al.*¹⁰ concerning requirements for histocompatibility for effective collaboration similarly used TNP-KLH in an equivalent *in vitro* system.

In conclusion, we can find no obligatory requirement for identity at the MHC for effective T-B collaboration in the

Table 3 Ability of histoincompatible T cells (H-2^d) to cooperate with H-2^k B cells

B cells	T cells	Antiserum treatment	Number of positive wells (30 wells assayed)	Total anti-TNP-PFC in 30 wells	% Dead T cells
CBA (H-2 ^k)	F ₁ (H-2 ^k × H-2 ^d)	None	30	4,464	10
	Chimaera	None	30	3,459	18
	H-2 ^k + H-2 ^d)	None	30	5,730	3
	CBA (H-2 ^k)	None	0	0	—
	No T cells	—	0	0	—
	CBA	Anti-H-2 ^d	30	4,046	3
	F ₁	Anti-H-2 ^d	13	578	58
	F ₁	Anti-H-2 ^k	5	179	85
	F ₁	Anti-H-2 ^k + H-2 ^d	2	25	95
	Chimaera	Anti-H-2 ^k	30	5,220	55
	Chimaera	Anti-H-2 ^k + H-2 ^d	0	0	93
CBA spleen cells irradiated with 1,200 r.	Chimaera	None	1	38	
	F ₁	None	1	36	
	CBA	None	2	40	

B cells and T cells were prepared as in Table 2. Antiserum treatment was as in Table 2 except that some chimaeric and F₁ T cells were treated with anti-H-2^k (1 ml 1:5) and anti-H-2^d (1 ml 1:2) sequentially before incubation with GPC. All cultures were challenged with 5 µg ml⁻¹ TNP-KLH and responses determined after 5 d. Viability data in this Table and in Table 2 confirm the chimaeric nature of the presumed chimaeric mice.

identical anti-H-2^k treatment. The confirmation that it was truly the remaining H-2^d T cells which were collaborating with the H-2^k B cells is shown in Table 3, in which the helper capacity of anti-H-2^k-treated chimaeric cells was totally abrogated by further treatment with anti-H-2^d alloantiserum. Furthermore, it was clear that the responding B cells were not contaminating syngeneic cells in the populations enriched with T cells, as these did not respond to TNP-KLH in the presence of irradiated CBA spleen cells. These data also indicate that the frequencies of helpers from H-2^d cells for H-2^k B cells must also be in the range found with the syngeneic cells.

Our data are in agreement with the recent *in vivo* findings of von Boehmer *et al.*¹⁴, who showed that SRBC-primed T cells from chimaeras would also cooperate with histoincompatible B cells for an anti-SRBC response on adoptive transfer, and also support the findings¹⁵ that

in vitro secondary immune response. This does not exclude, however, any more subtle requirement for MHC identity between cells for the generation of efficient helpers.

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Low dose unresponsiveness with a thymus independent antigen

LIPOLYSACCHARIDES (LPS) from Gram-negative bacteria are very efficient carriers for haptenic determinants, so that B cells can be activated by such conjugates to produce antihapten responses both *in vivo* and *in vitro* in the absence of T helper cells¹⁻³ and macrophages (our unpublished data). Trinitrophenylated LPS (TNP-LPS) is one such thymus-independent antigen. Most antigens of this category can induce unresponsiveness in animals only when administered in high concentrations⁴, unlike certain thymus-dependent antigens which can induce unresponsiveness *in vivo* in both low doses (low zone unresponsiveness) and high doses (high zone unresponsiveness)⁵. In the case of low zone unresponsiveness to thymus-dependent antigens, only helper T-cell functions seem to be affected⁶. It

Table 1 T-cell independence of unresponsiveness

Groups*	PFC per 10 ⁷ spleen cells	
	<i>nu/nu</i>	Hairy littermates
Controls	4,783	7,292
Preinjected with LPS (0.07 µg)	390	353

Spleens from seven *nu/nu* and five littermates were each assayed in duplicate. Arithmetic means are shown.

*Mice were injected with 5 µg of TNP-LPS 5 d after injection of water (controls) or 0.07 µg LPS.

was therefore interesting when we observed that very low doses of LPS could render mice unresponsive to subsequent TNP coupled to LPS. Only in one previous case has low zone unresponsiveness to a thymus-independent antigen been observed, and this was with pneumococcal polysaccharide (SSS III) through a mechanism involving suppressor T cells⁷. We report here that low zone unresponsiveness achieved with LPS is highly specific and requires no obvious T-cell-mediated suppressor mechanisms, thus indicating a further type of regulation mechanism for antibody responses. As LPS are intimately related to other

bacterial surface antigens it may be that similar mechanisms operate in the determination of natural immunological reactions against Gram-negative bacteria.

CBA, C3H, C3H/HeJ, BALB/c and BALB/c *nu/nu* mice, bred in the Department of Pathology, Cambridge were used. Recipients for transferred spleen cell inocula were irradiated with 675 rad from a Co source. Trinitrophenylated (TNP) derivatives of these LPS (*Escherichia coli* 055:B5) and LPS (*Serratia marcescens*) were obtained from Difco and were prepared by the technique of Jacobs and Morrison¹. Dinitrophenylated (DNP)-dextran was a gift from Dr C. Desaymard. All injections were given intraperitoneally, and all plaque-forming cells (PFC) were assayed by conventional techniques. Anti-TNP responses were measured at the optimal time, 3 d after challenge,

Table 3 Abrogation of unresponsiveness after lymphocyte transfer

Pretreatment of donors of nude spleen cells	Pretreatment of irradiated recipient mice*	TNP-PFC per spleen†
None	None	10,900
LPS (0.07 µg) on day -5	None	12,040
None	LPS (0.07 µg) on day -5	200

*Heterozygous BALB/c littermates were irradiated with 600 rad. Nude spleen cells (40 × 10⁶) were then transferred to recipients. TNP-LPS (*E. coli*) (5 µg) were injected 2 h later.

†Each value represents the geometric mean of PFCs per spleen from four mice each assayed in triplicate.

except where stated otherwise. Initial experiments showed that when BALB/c mice were injected with various doses of LPS (*E. coli*) 5 d before challenge with TNP-LPS as little as 7 × 10⁻⁴ µg LPS could diminish the subsequent anti-TNP response to 36% of the control level and 7 × 10⁻² µg effectively abolished it (our unpublished data). This effect could be seen 72 h after treatment with 7 × 10⁻² µg LPS and was complete by 96 h (our unpublished data). The phenomenon does not obviously depend on any T-cell suppressor mechanisms as it is seen in nude mice (Table 1), but it is carrier specific in that none of the responses to DNP-dextran, TNP-LPS (*S. marcescens*) or sheep red blood cells (SRBCs, Table 2) were affected. This suggests that the specificity of the unresponsiveness is related to the polysaccharide portion of O antigens of LPS as these seem to be the immunodominant determinants.

The status of TNP-specific B cells seemed to be unaffected by LPS (*E. coli*) because spleen cells from treated groups responded as well as untreated controls in adoptive transfer. In contrast mice pretreated with LPS (*E. coli*)

Table 2 Specificity of low dose unresponsiveness

Treatment of mice (CBA)	DNP-Dextran	PFC per spleen from challenge with		TNP-LPS (<i>S. marcescens</i>)
		TNP-LPS (<i>E. coli</i>)	SRBCs	
<i>Experiment 1</i>				
LPS (<i>E. coli</i> 055: B5) (0.07 µg) on day -5	21,000	1,000	17,720	NT
Nil	21,050	74,000	11,140	NT
<i>Experiment 2</i>				
Nil		50,000*		33,150*
LPS (<i>E. coli</i>) (0.07 µg) on day -5		3,970*		41,110†

DNP-Dextran (2.5 µg) was given 4 d before TNP-PFCs were assayed. TNP-LPS (2.5 µg) was given 3 d before TNP-PFC were assayed. SRBCs (0.2 ml, 5%) were given 4 d before SRBC-PFC were assayed. Figures represent the geometric mean of the response of three to six spleen donors. NT, not tested.

*TNP-LPS (10 µg) was injected into each mouse. TNP-PFC were assayed 3 d later.

†Each value represents the geometric mean of PFCs per spleen from four mice assayed in triplicate.

were poor recipients for adoptively transferred normal spleen cells in that they responded poorly to TNP-LPS (*E. coli*) but normally to TNP-LPS (*S. marcescens*) (Table 3). Further unpublished data show that LPS non-responder mice (C3H/HeJ) pretreated with LPS behave in the same way as untreated recipients for normal cells (that is they produced no suppression). Therefore, unresponsiveness must be related to a lipid A-dependent activation event⁸⁻¹⁰, the most likely of which could be the production of anti-LPS antibody, albeit in very small amounts.

In conclusion our data demonstrate that a highly immunogenic thymus-independent antigen, LPS, generates, on low dose priming, an LPS-specific product which prevents the successful interaction of TNP-LPS with hapten-specific cells. We predict that this phenomenon should not be exclusive to LPS but should be detectable with other strongly immunogenic thymus-independent antigens.

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Effect of tumour necrosis factor on cultured human melanoma cells

CARSWELL *et al.*¹ described a tumour necrosis factor (TNF) in the sera of mice sensitised with BCG and injected with endotoxin. After intravenous injection of this serum into mice bearing a 7-d-old transplant of a tumour originally induced by methylcholanthrene, necrosis of the tumour began within 12-24 h. A similar response was found in mice bearing various other tumours. We have now found that both whole TNF serum and TNF partially purified from mouse serum² inhibit the growth of a human melanoma cell line.

We have screened neuroblastoma and melanoma cell lines to determine their susceptibility to TNF. We used these tumours because they have a high degree of spontaneous³ or immune-induced regression⁴. They had all been maintained in continuous culture in our laboratories and consisted of a melanoma (RPMI 7931) originally established by George E. Moore, Roswell Park Memorial Institute and maintained free of mycoplasma by Dr Jørgen Fogh, Memorial Sloan-Kettering Cancer Center; three neuroblastoma lines, two described previously⁵, and a fibroblast line derived from the bone marrow of a child with neuroblastoma.

TNF in serum or after partial purification² (fraction G-200 II) had no growth inhibitory or toxic effects on neuroblastoma or fibroblast cells. These observations agree with those of Carswell *et al.*¹, who found that normal mouse embryo fibroblasts (MEF) were insensitive to TNF. In contrast, growth of melanoma cells was inhibited (Table 1). At the highest level of TNF activity, that is where the concentration of protein was 0.45 mg ml⁻¹ or more, cell proliferation stopped. When the concentration of TNF was decreased, the extent of inhibition of cell growth was

Table 1 Effects of TNF on growth *in vitro* of melanoma cells (RPMI 7931)

Substance added to culture medium	Mouse serum protein in culture medium (mg ml ⁻¹)	Inhibition of growth (%)
(1) None	0	0
(2) Serum (untreated mice)	7.0	0
(3) Serum (endotoxin-treated mice)	7.0	0
(4) Serum (BCG-treated mice)	7.0	0
(5) Serum (BCG + endotoxin-treated mice)	7.0	100
(6) G-200 II from sera of normal mice	0.2	0
(7) G-200 II from sera of BCG + endotoxin-treated mice (TNF)	0.05	10
(8) G-200 II from sera of BCG + endotoxin-treated mice (TNF)	0.1	10
(9) G-200 II from sera of BCG + endotoxin-treated mice (TNF)	0.2	75*
(10) G-200 II from sera of BCG + endotoxin-treated mice (TNF)	0.45	100
(11) G-200 II from sera of BCG + endotoxin-treated mice (TNF)	1.0	100
(12) None*	0.0	80

*Cells from this culture were replated without additional TNF (see line 12).

G-200 II represents a 20-fold purification of TNF².

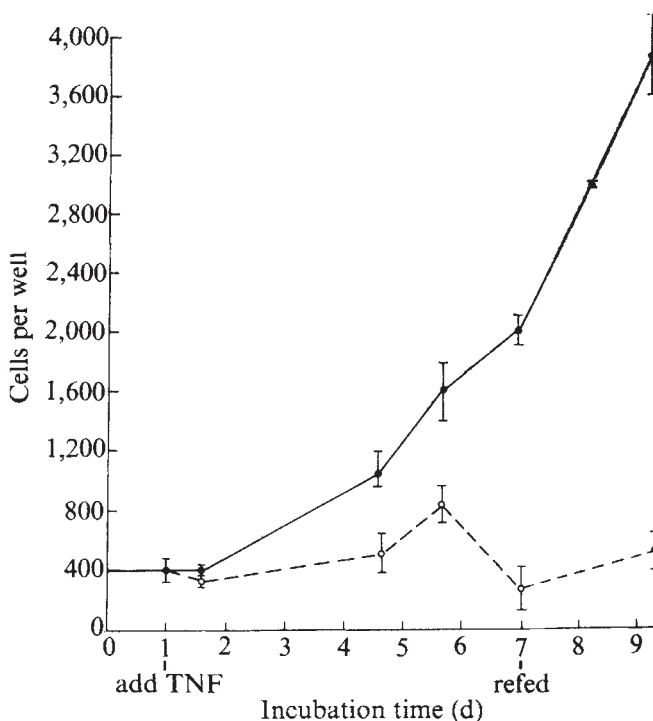


Fig. 1 Melanoma cells were grown in 25-ml plastic flasks (Falcon) containing 7 ml of Eagle's medium with glutamine, non-essential amino acids, penicillin, streptomycin and 15% heat-inactivated foetal bovine serum. Cultures were incubated at 37 °C in a 5% CO₂ atmosphere. Stock cultures were grown to confluence and refed with fresh medium, 24 h later the cells were detached with 0.25% trypsin and 500-1,000 cells in 0.1 ml were inoculated into wells of a 96-well flat bottomed microtitre plate. To each test well 0.06 ml of a solution of TNF was added. The final concentration of the TNF protein per ml of incubation mixture ranged from 0.05 to 1.0 mg. At 2-3-d intervals the number of cells from three control and three TNF-treated wells were determined as follows. The well contents were removed, each well was washed twice with phosphate-buffered saline and 0.2 ml of a 0.25% trypsin solution was added to detach adhering cells. After 15 min the total number of cells in the suspension, ranging in size from 80 to 280 µm, was counted with a Coulter counter. ●, Normal medium; ○, medium plus TNF (1.0 mg per ml⁻¹ mouse serum protein).

reduced. Inhibited cells which were replated in fresh media without additional TNF (Table 1, lines 9 and 12) did not return to their normal growth rate. The kinetics of melanoma cell growth in the presence or absence of TNF are shown in Fig. 1.

This inhibition of human melanoma cells by partially purified TNF was not due to nonspecific cytotoxic material present in mouse serum, for crude and identically prepared fractions from normal mouse serum lacked growth inhibitory activity. The persistence of growth inhibition after replating suggests that (1) the factor might remain bound to the cell; (2) plating efficiency is diminished secondarily to structural alterations in the cell membrane caused by contact with TNF, or (3) TNF induces metabolic alterations within the cell.

In summary, the combination of BCG sensitisation and endotoxin challenge in mice results in the production and release into their circulation of a substance which is cytotoxic to cultured human melanoma cells.

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4a(W4) and 4b(W6) human histocompatibility antigens are specifically associated with complement receptors

THE biological role of the histocompatibility genes is currently the subject of intense research and speculation¹⁻³. It has been shown that efficient cell cooperation and thus immune response requires H-2 (I region) compatibility⁴ and that some components of the complement (C) system, including complement receptors (CR) which may have an important role in cell-cell interaction and in lymphocyte cooperation^{5,6} are also under the control of the MHS both in mouse⁷ and man^{8,9}. In mouse, we have shown that the CR polymorphism is partly controlled by genes mapping in the I-C or S region of the H-2 complex¹⁰. Now, in man, we show also that the cell surface gene products of the diallelic human HLA 4a, 4b system¹¹ (also named W4 and W6, respectively)—now established to be an independent¹² genetic system inside the HLA complex and whose gene products are believed by some to be HLA-B basic¹² or supertypic¹³ specificities—could form an integral part of complement receptors. In short anti-4a and anti-4b antisera (or their F(ab')₂ fragments) are able specifically to block the rosetting capacities of complement receptors on 4a and 4b positive lymphocytes respectively (that is with antibody (A) and

complement (C)-coated sheep red blood cells (E), (EAC)).

Blood samples were obtained from normal people and some chronic lymphatic leukaemia patients who had previously been typed for HLA-A, -B, -C and 4a, 4b antigens. The blood was defibrinated, layered on Ficoll-Trisil and centrifuged. The lymphocyte band was removed and washed in veronal buffer solution (VBS); the cells were resuspended in VBS, subsequently divided into aliquots and preincubated with anti-HLA-A, -B and -C antisera and anti-4a, 4b antisera or their F(ab')₂ fragments. After washing twice in VBS, complement receptors were assayed by a modification of the rosetting method described in ref. 14. The rosettes were counted under a fluorescent microscope after the addition of 0.01% acridine orange to the suspensions. Pretreatment of homozygous 4a cells with anti-HLA-A -B and -C antibodies (Table 1) and anti-4b sera did not affect the number of complement rosetting cells (CRC). In contrast, both the anti-4a antisera used inhibited by 60-70% the number of complement rosettes formed. Similar results were obtained using anti-4b sera with homozygous 4b cells. The number and variety of HLA antisera and the various cell types used make it unlikely that serum activity other than anti-4a, 4b antibodies was responsible for CR inhibition. The results with 4a/4b heterozygous lymphocytes were more complex. Complement rosettes made with these cell suspensions (except with BR lymphocytes) were more easily inhibited by anti-4b, than anti-4a sera. Since by cytotoxic definition most heterozygous 4a/4b individuals' lymphocytes express both 4a and 4b antigens; because of the difference in susceptibility to inhibition by the anti-4a, anti-4b antisera by different individuals' lymphocytes, we find that it is possible to subdivide the heterozygotes as follows: (1) those with CR only inhibited by anti-4b (BW; KH); (2) those with CR most readily inhibited by anti-4a, but also significantly inhibited by anti-4b (BR) and (3) those with CR most readily inhibited by anti-4b, but also significantly with anti-4a (CB, JP). When anti-4a and anti-4b were both used at the

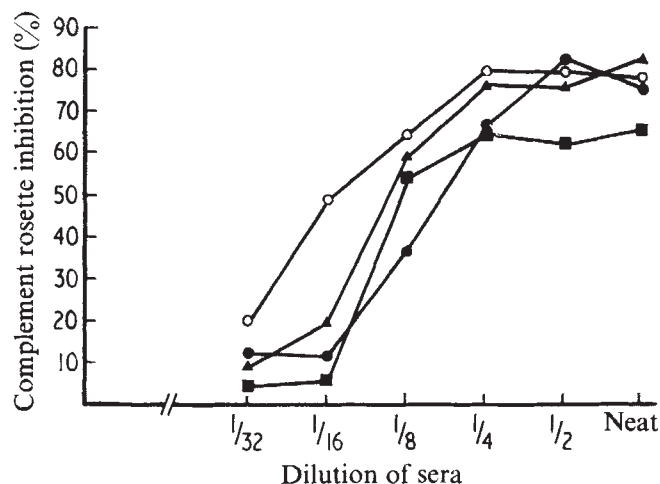


Fig. 1 For titration of anti-4a sera, AA-V cells were used; for titration of anti-4b sera, MW cells were used (see Table 1). The working dilutions used throughout were: for both anti-4a (1:2); for both anti-4b (1:4). Lower dilutions produced cell agglutination. These particular 4a, 4b antisera were characterised as highly specific and "extra" reactions were not found with our cell panel. Inhibition (%): [1-(% rosettes after pretreatment with human AB Rh negative serum / % rosettes after pretreatment with test sera)] × 100. The percentage of rosettes after pretreatment with human AB Rh negative serum was not modified. ▲, F(ab')₂ anti-4a (van Rood, 12123.2) which gives a titration curve similar to the whole serum = 4a₁ (not represented); ■, whole serum anti-4a (Claudia, Balner) = 4a₂; ○, whole serum anti-4b (van Rood, 9097.1) = 4b₁; ●, whole serum anti-4b (Hinchcliffe, Morris) = 4b₂.

Table 1 Inhibition* of complement receptors by human anti-W4(4a) and anti-W6(4b) sera

Cell typing†	CRC %	CRC inhibition % (numbers in parentheses) after pretreatment with antisera anti — :						
		First series (HLA-A)	Second series (HLA-B)	Third series (HLA-C)	4a ₁ ‡	4a ₂	4b ₁	4b ₂
AA—V (1, 29/12, W17/4a, 4a)	19	A1(3), A29(0.)	B12(0.), BW17(—2)	—	(64)§	(60)	(3)	(0.)
BG (2, W26/12, W21/4a, 4a)	28	A2(0.), AW26(0.)	B12(1.), BW21(5)	—	(74)§	(76)	(1)	(0.)
PH (1, 2/7, 8/4b, 4b)	26	A1(0.), A2(0.)	B7(0.), B8(0.)	—	(1)	(10)	(65)	(67)
MW (2, X/W35, 8/4b, 4b)	22	A2(0.)	BW35(0.), B8(10)	—	(6)	(8)	(68)	(72)
BT (2, 3/W35, W40/T2, T4/4b, 4b)	24	A2(0.), A3(0.)§	BW35(—0.), BW40(6)	T2(0.)§, T4(0.)	—	—	(50)	(79)
MH (1, X/8, W35/T4/4b, 4b)	18	A1(0.)	B8(0.), BW35(0.)	T4(0.)	—	—	(66)	(70)
LG (W26, 10.4/14, W35/4b, 4b)	22	AW26(0.), A10.4(3)	B14(0.), BW35(0.)	—	—	—	(70)	(70)
BW (W25, W32/W16, 18/T3/4a, 4b)	16	AW25(0.), AW32(0.)	BW16(0.), B18(0.)	T3(0.)	(1.)§	(1.)	(60)	(68)
CB (2, X/12, W15/T3, T4/4a, 4b)	26	A2(0.)	B12(0.), BW15(0.)	T3(0.)	(30)§	(20)	(54)	(63)
BR (2, 11/5, W35/T1, T4/4a, 4b)	19.5	A2(5), A11(9)	B5(0.)§, BW35(6)	—	(77)§	(71)	(46)	(40)
JP (1, 3/7, W37/4a, 4b)	19	A1(0.), A3(0.)§	B7(0.), BW37(0.)	—	(31)	(30)	(78)	(82)
KH (3, 11/12, W40/4a, 4b)	21	A3(1)§, A11(—1)	B12(0.), BW40(1)	—	(0.5)	(0.)	(50)	(61)
Chronic lymphocytic leukaemia								
Hew. (2, X/W17, 15T1/4b, 4b)	60	A2(—2)	BW17(0.)	—	—	—	(94)	—
Dough. (1, 11/8, 12/4a, 4b)	65	A1(0.)	B8(0.)	—	(80)	—	(40)	—

All anti HLA antisera were used 1:2 dilution; anti-4a₁, —4a₂, —4b₁, —4b₂ as specified in Fig. 1. 2×10^6 VBS washed peripheral leukocytes were incubated 45 min at room temperature with 20 μ l of the antisera in precipitin tubes. The cells were washed twice in VBS and sheep red blood cells covered by antibody and complement (EAC) were added (37 EAC: 1 lymphocyte ratio), spun down, incubated 8 min at room temperature, resuspended and the complement rosette percentage immediately counted. EA controls were 2–3% and E were negative throughout.

Antisera were spun down at 14,000g for 5 min before use.

* Inhibition % as Fig. 1.

† July 1975 new WHO nomenclature for HLA system, except that T1 = HLA—CW1, T2 = HLA—CW2, T3 = HLA—CW3, T4 = HLA—CW4.

‡ See Fig. 1.

§ F(ab')₂ fragments of HLA antisera were used at 1:2 dilution or according to Fig. 1 plateau dilution values in the case of 4a₁. Whole sera gave substantially the same inhibition number, as they were also used in parallel.

same time for preincubating with BR cells, there was still only 73% inhibition (71% with anti-4a alone; 40% with anti-4b alone), indicating that one particular CR may be associated with both 4a, 4b products. These findings suggest that there may be different types of 4a/4b heterozygous gene products which might also be influenced by epistatic interaction with genes outside the MHS.

The inhibition of complement rosette formation was invariably partial: about 30% of rosettes were not inhibited. We believe this could be due to a specific reaction with lymphoid and not myeloid CR because the percentage fits with the number of non-lymphoid CRC in human peripheral blood¹³ and the higher CR inhibition obtained when

testing chronic lymphocytic leukaemia lymphocytes (Table 1). This conclusion is in agreement with our previous results in mice¹⁶, where anti-H-2 sera inhibited only lymphoid complement receptors.

Only CR were specifically inhibited by anti-4a and anti-4b antisera. Sheep red blood cell (T) rosettes were not inhibited at all and Fc rosettes were nonspecifically inhibited by all the anti-HLA sera tested (Table 2) as anti-H-2 antisera behaved in the mouse¹⁷; nor did preincubation with rabbit anti human Ig affect CR numbers (unpublished).

Our results therefore suggest that the 4a, 4b gene products if not identical with CR may either constitute an integral part of them or be very closely associated with them. HLA—

Table 2 4a and 4b are not specifically associated with receptors other than complement receptors

Cell typing†	Anti-HLA sera used for inhibition (rosette inhibition (%))	
	Fc rosettes§	SRBC rosettes
AA-V	C1(27), ‡ A1(60), A29(67)*, B12(70), BW17(70), 4a ₁ (69)*, 4a ₂ (70).	C1(61), A1(0.), A29(—2), B12(0), BW17(1), 4a ₁ (—2), 4a ₂ (0).
BT	C1(28), A2(71), A3(70)*, BW35(72), BW40(73), T2(77)*, 4b ₁ (74), 4b ₂ (70).	C1(50) A2(0), A3(0.), BW35(0.), BW40(1), T2(0.), 4b ₁ (0.), 4b ₂ (0).
BR	C1(24), A2(71), A11(70), B5(71)*, BW35(70), T4(71), 4a ₁ (60)*, 4b ₁ (63).	C1(60). A2(0.), A11(0.), B5(0.) BW35(—2), T4(0.), 4a ₁ (0.), 4b ₁ (0.).

Values showed are mean of two different experiments.

* In these cases, F(ab')₂ fragments were also used.

† See Table 1.

‡ Underlined are the % rosettes in controls, see Fig. 1.

§ 50 μ l 1% ox cells in VBS covered by rabbit anti-ox IgG (provided by Dr M. B. Pepys) were mixed with 50 μ l of 2×10^6 lymphocytes per ml in VBS in Precipitin tubes. They were spun down and counted straight away.

|| 0.5 ml fresh 5% sheep red blood cells in VBS were mixed with 0.5 ml 5×10^6 per ml lymphocytes in VBS; prewarmed 5 min at 37 °C, spun down and incubated at 4 °C for a further hour. Rosettes were counted after careful resuspension.

A, -B and -C gene products are expressed in different molecules on the cell surface (that is they cap independently, see review in ref. 18). Using these latter methods, we and others^{19,20} have not been able to dissociate 4a/4b from HLA-B cell membrane gene products. Nevertheless, anti-4a and anti-HLA-B sera behave differently in our present experiments and in addition we found that 4a and 4b²¹ have different linkage disequilibria with different HLA-B antigens in Negroid and Caucasoid populations, supporting the concept of separate genes controlling HLA-B and 4a/4b surface determinants. Furthermore, recent results of 50 diverse population studies of 4a, 4b have shown that they fit the Hardy-Weinberg equilibrium¹². Also, interestingly enough, the gene frequencies (0.35 for 4a and 0.65 for 4b)¹² approximate to that for human C3 in North American Caucasoids (C3': 0.22 and C3'': 0.77)²². C3' and C3'' also have different binding capacities for complement receptors²³. Our EAC (prepared with mouse complement) attach chiefly to the C3d receptor²⁴ of lymphocytes²⁴ and certain myeloid cells²⁵ and any attempt to relate the polymorphism of C3 to that of CR will have to take into account that we are probably detecting an inhibition of C3d receptors on lymphocytes. Although we have already shown that I region (I-C/S) products and CR are associated in the mouse^{19,16}, we are not yet in a position to make any direct comparisons with man but experiments designed to investigate the possible relationship between Ia antigens and 4a, 4b antisera activity are in progress. Furthermore, the presence of anti-4a, 4b antibodies in pregnant women might affect (deleteriously?) foetal complement receptors.

In conclusion, these experiments strongly support the single-gene theory for the 4a, 4b system and also give an indication of a direct physiological role for an HLA antigen.

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Identification of a new oncofoetal antigen associated with several types of human carcinomas

In experimental animals, oncofoetal antigens¹ have been found to be associated with both chemical² and virus-induced tumours³. In man the two best known oncofoetal antigens are the α -foetoprotein (AFP) described by both Abelev⁴ and Tatarinov⁵ and the carcinoembryonic antigen (CEA) of the human digestive system identified by Gold and Freedman⁶. We describe here a different human oncofoetal antigen, common to several types of carcinomas and various foetal organs. This antigen has been identified by rabbit antisera raised against semipurified fractions of colon carcinoma soluble extracts. Because of its β -immunoelectrophoretic mobility, this antigen will be referred to as β -oncofoetal antigen (BOFA).

Primary tumours and adjacent normal tissues were obtained from surgical excisions, metastatic tumours from autopsies and foetal organs from spontaneous or therapeutic abortions. Soluble extracts of these tissues were prepared by a standard procedure: 0.2–0.5 cm³ fragments were suspended in two volumes phosphate buffer (0.03 M; pH 7.4) and homogenised in a Sorvall Omni mixer at 15,000 r.p.m. for 30 min at 4 °C. After centrifugation of the homogenate at 30,000g for 30 min, the supernatant fluid was dialysed against 20 volumes deionised water for 24 h at 4 °C, and lyophilised. This extraction procedure yielded 40–50 mg dry powder per g wet tissue.

The extract from a large hepatic metastasis of a colon carcinoma was fractionated on Sephadex G-200. Each of four selected fractions was applied on a DEAE-cellulose column equilibrated with phosphate buffer (0.01 M; pH 8) and eluted stepwise with buffer of increasing molarity and decreasing pH. Twenty rabbits were immunised by repeated injections of 3 mg of the various DEAE-cellulose fractions in Freund's complete adjuvant. The three rabbits which received the colon carcinoma fraction eluted from Sephadex G-200 between the IgG and the serum albumin peak and then from DEAE-cellulose after the second buffer (phosphate 0.02 M; pH 7.4) produced the antisera which, after absorption with normal tissue extracts and plasma, became specific for BOFA. Every millilitre of antiserum was absorbed with 20 mg each of soluble extract from normal colon mucosa, normal liver, normal spleen, normal lung, AB Rh⁺ red cells and with 0.2 ml normal plasma.

In double diffusion the absorbed anti-BOFA antiserum revealed a single precipitin line with whole colon carcinoma extract but gave no lines with extracts of normal organs or with normal plasma (Fig. 1a). This antiserum gave a line of identity between extracts from carcinomas of the colon, lung, breast, liver, pancreas and from melanomas (Fig. 1b) as well as with extracts from the intestine, lung, liver and kidney of a human foetus of 16 week gestation (Fig. 1c). In all 34 carcinoma extracts (16 primary tumours and 18 hepatic metastases) and all 31 foetal organ extracts listed in Table 1 (with the exception of foetal skin), the same precipitin line was observed. In contrast, all 29 normal tissue extracts listed in Table 1, obtained from 14 different individuals, gave negative results, even at concentrations as high as 300 mg lyophilised powder ml⁻¹. Thus, at the level of sensitivity of the double diffusion test, BOFA seemed to be oncofoetal specific⁷.

BOFA was also identified in extracts of human tumours (four colon carcinomas, four melanomas, two endometrial carcinomas) maintained by serial transplantation in nude mice^{8–10} and in extracts of human tumour cell lines which had been established in tissue culture for several months or years. Controls using extracts of normal mouse tissue or

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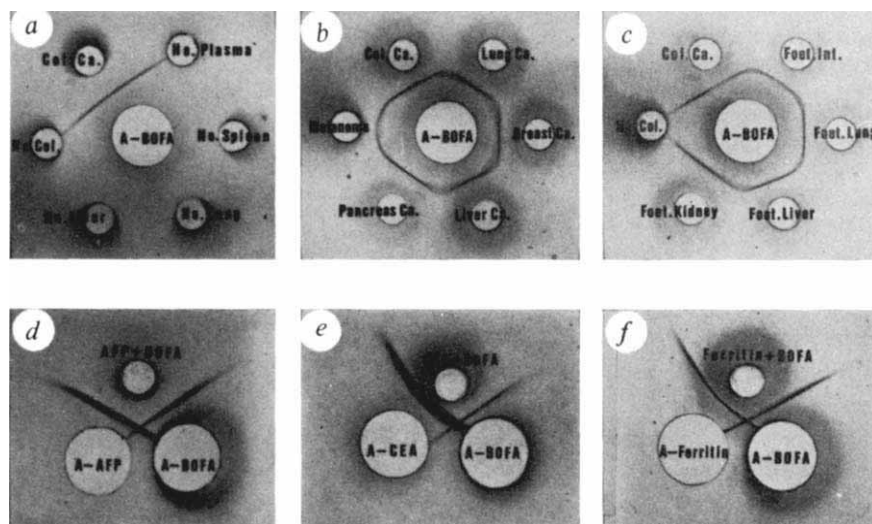
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Fig. 1 Double diffusion in agar showing presence of BOFA in extracts of human tumoral and foetal tissues. *a, b, c*, Central wells: specific rabbit anti-BOFA serum absorbed with normal tissue extracts; antigens: *a*, colon carcinoma, normal neat plasma, normal spleen, normal lung, normal liver, normal colon; *b*, carcinoma of colon, lung, breast, liver, pancreas and melanoma; *c*, colon carcinoma, intestine, lung, liver and kidney of a foetus of 16 weeks' gestation, normal colon. All the antigens are whole lyophilised soluble extracts of tissues at a concentration of 100 mg ml⁻¹. *d*, Left well: specific anti-AFP serum (from Dr Sizaret, IARC Lyon France); right well: anti-BOFA serum; upper well: mixture of AFP and BOFA. *e*, Left well: specific anti-CEA serum (obtained as described previously¹¹); right well: anti-BOFA serum; upper well: mixture of CEA and BOFA. *f*, Left well: specific anti-ferritin serum (from Behringwerke); right well: anti-BOFA serum; upper well: mixture of ferritin and BOFA.



concentrated culture medium containing foetal calf serum but no tumour cells gave no precipitin lines. These results indicate that BOFA is synthesised by the tumour cells.

BOFA is immunologically different from AFP, CEA or ferritin, as demonstrated by the crossed precipitin lines shown in Fig 1*d-f*. Ferritin was tested because antigens immunologically identical to ferritin, described as α -2H (ref. 12) or iso-ferritin^{13,14}, have been shown to possess oncofoetal properties. BOFA is also distinct from the normal glycoprotein (NGP) cross reacting with CEA¹⁵ and the β -2-microglobulin¹⁶. Four other oncofoetal antigens described in the literature also seem to be different from BOFA. The γ -foetoprotein¹⁷ and the leukaemia-associated antigen¹⁸, unlike BOFA, were both detectable in foetal sera by the conventional double diffusion test. The antigen described by Klavins *et al.*¹⁹ was detected in extracts of adult skin and was reported to have an isoelectric pH of 4.8 (ref.

20). The β S-foetoprotein was reported to be soluble in 0.6 M perchloric acid and to be eluted with the exclusion volume of Sephadex G-200 column. These properties differ from those of BOFA. Using immunoelectrophoresis, BOFA has been shown to have a β -2-mobility (Fig. 2*a*), and using isoelectric focusing on thin-layer polyacrylamide gel, a pH, ranging from 6 to 7. According to its elution on Sephadex G-200, BOFA has a molecular weight of 70–90,000. BOFA can be precipitated by 0.1 M perchloric acid and 33% saturated ammonium sulphate. Its antigenicity can be abolished by treatment with Pronase or by incubation at 70 °C for 30 min. In a caesium chloride gradient, the density of BOFA ranges between 1.30 and 1.32. These properties suggest that BOFA is a protein which does not contain a large amount of carbohydrate or lipid. Partial purification of BOFA was achieved by adsorption-elution from an immunoadsorbent

Table 1 Concentration of BOFA in various human tissues

Tissues	Tumour	Foetus	Normal
Colon and rectum	Primary: 165(80–320)* [8]†	88(75–120) [5]	4.0(2.5–5.0) [9]
	Metastatic: 303(83–483) [9]		
Lung	Primary: 76(66–83) [2]	60(40–66) [5]	3.0(1.0–3.3) [4]
	Metastatic: 106(87–120) [3]		
Liver	118(75–160) [3]	170(160–183) [5]	5.0(2.5–7.5) [4]
Kidney	47(42–50) [3]	140(83–160) [5]	4.1(3.3–5.0) [3]
Pancreas	80(66–91) [3]	83(73–93) [3]	NT
Breast	70(50–85) [3]	NT	2.6(2.0–3.6) [3]
Brain	NT	48(40–53) [5]	1.5(1.3–1.8) [3]
Skin	NT	18(15–27) [3]	1.3(1.0–1.5) [3]

*Mean and upper and lower limits in μ g units per 100 mg lyophilised soluble extracts.

†Number of specimens tested.

NT, not tested.

Table 2 Detection of BOFA in various human cell lines

Origin	Cell line	Concentration of BOFA*	Surface immuno-fluorescence†
Colon carcinoma	HT29‡	50	++
	Col15§	66	+++
	Col125§	60	++
Breast carcinoma	SK-Br 3‡	60	++
	BT20	70	+++
Melanoma	SK-Mel 1‡	60	++
Endometrial carcinoma	END1§	66	++
Cervix carcinoma	Me-180‡	25	++
	HeLa¶	6	—
Burkitt lymphoma	Raji¶	3	—
Lymphoblastic leukaemia	Molt-4¶	0	—
Normal lymphocytes	Lik¶	0	—

* μg units per 100 mg lyophilised soluble extract.

†As determined by staining anti-BOFA antiserum treated living cells with fluoresceinated sheep anti-rabbit IgG conjugate.

‡Obtained from the Sloan-Kettering human tumour cell line bank, New York.

§Established by Dr S. Carrel in our Institute.

||Obtained from Professor L. Ozzello, Institute of Pathology, Lausanne.

¶Obtained from the Swiss Institute for Experimental Cancer Research, Lausanne.

consisting of IgG from a specific rabbit anti-BOFA antiserum, coupled to Sepharose 4B. This method yielded small amounts of BOFA which were not sufficiently pure for use in a radioimmunoassay, but could be used as a preliminary reference antigen preparation for the quantitation of BOFA in different tissue and plasma samples.

As the conventional single radial diffusion test²² was not sensitive enough to detect BOFA in patients' sera, we used

the modification described by Rowe²³ in which the area of antigen-antibody precipitate is revealed by autoradiography after incubation of the plates with ¹²⁵I-labelled sheep IgG anti-rabbit IgG. By this method, which is about 30 times more sensitive than the conventional Mancini plates²², it was possible to demonstrate the presence of BOFA in normal adult tissues as well as in the plasma of foetuses, of cancer patients and, at a slightly lower level, in the plasma of normal adults. The immunological identity between the small amount of BOFA present in various plasma samples with the reference BOFA from colon carcinoma extract was demonstrated by the radioactive double diffusion test developed by Abelev²⁴, using diluted reagents and a radio-labelled second antibody (Fig. 2b, c).

Results of the quantitative study of BOFA in various tissues, expressed in units of BOFA (corresponding to about 1 μg) per 100 mg lyophilised saline are shown in Table 1. The highest concentrations of BOFA were found in primary and metastatic carcinomas of the large bowel and in foetal liver and kidney. The concentration of BOFA was also relatively high in the 10 foetal placentae tested (mean, 101; range, 46–170), whereas in the placentae at the end of gestation it was only slightly lower (mean, 69; range, 40–80). In normal adult tissues the BOFA concentrations were 10–75 times lower than in the corresponding tumours. The most significant difference in BOFA concentration was observed by comparing the extract of a primary colon carcinoma, containing 320 units, with an extract of the normal colon mucosa, containing 2.5 units BOFA, both originating from the same patient.

In spite of the differences in tissue concentration, the plasma levels of BOFA in cancer patients and foetuses were not much higher than those in normal individuals. The quantitative study of BOFA in plasma samples from normal individuals and patients gave the following results (μg units ml^{-1} , mean and range): in 60 unselected blood bank donors, 1.0 (not detectable–3.4); in 34 patients with non-neoplastic inflammatory bowel diseases or liver cirrhosis, 2.5 (not detectable–5.1); among 57 patients with large bowel carcinomas, 18 with localised tumours (Dukes A and B), 5.8 (3.6–8.6); 22 with lymph node infiltration (Dukes C), 5.4 (3.8–8); 17 with distant metastases (Dukes D), 5.6 (4.0–7.2);

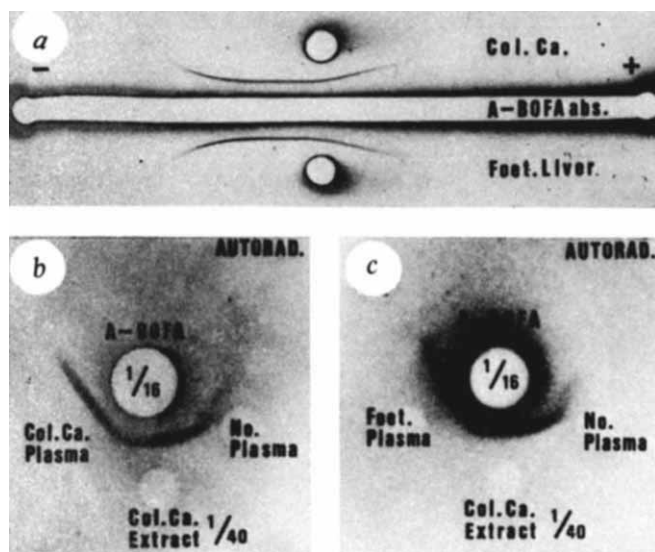


Fig. 2 a, Immunoelectrophoretic analysis of a whole colon carcinoma extract (Col. ca.) and a whole foetal liver extract (foet. liver). Antiserum: A-BOFA abs., rabbit anti-BOFA serum absorbed with normal tissues and plasma as described in the text. b and c, Autoradiographs of radioactive double diffusion in agar showing the immunological identity between BOFA present in colon carcinoma extracts (middle lower well, b and c) and the small amount of BOFA present in the plasma of a colon carcinoma patient (left well, b), a foetal plasma (left well, c) and a normal plasma pool (right well, b and c). Absorbed anti-BOFA antiserum was diluted 1/16, the colon carcinoma extract was at a concentration of 2.5 mg ml^{-1} (diluted 1/40 compared with Fig. 1a–c), whereas the three plasma samples are undiluted.

in 40 patients with various other types of carcinomas as well as melanomas and lymphomas, 5.4 (3.8–7.2). Elevated BOFA levels did not seem to be limited to a particular type of cancer. BOFA was measured before and after operation in the plasma of 16 patients who had a complete surgical resection of a large bowel carcinoma and whose CEA level dropped significantly or remained low after surgery²⁵. In these patients the BOFA levels showed a moderate decrease 1–3 months after tumour removal. In 16 of these cases the preoperative values were 5.1 (3.8–6.6), whereas the lowest postoperative values were 1.9 (0.5–3.8). In cases of tumour recurrence²³, BOFA levels increased moderately in some, but not in all patients. It is too early to decide whether BOFA determination may have a clinical interest in connection with assays of other tumour-associated antigens such as CEA^{25–28} and AFP²⁹.

The BOFA values were 6.3 (5.1–8.2) in the plasma of five fetuses of 14–20 week gestation, and remained relatively high at the end of gestation in 20 cord plasma samples tested: 8.2 (6.6–11). BOFA levels seemed to decrease progressively during the first 6 months of life, reaching values of 2.0 (not detectable–4.6) in the 30 plasmas of children aged 6 months–2 yr tested.

From the comparison between BOFA concentrations in tissues and in plasma, it seems that this antigen is not actively secreted by foetal or tumour cells. To determine the cellular localisation of BOFA, several human carcinoma or lymphoid cell lines were tested by indirect immunofluorescence. As shown in Table 2, BOFA could be demonstrated on the surface of eight of the nine carcinoma cell lines tested, whereas the fluorescence was negative on HeLa cells and on the three lymphoid cell lines, which contained little or no detectable antigen in their soluble extracts.

The functional role of this antigen remains to be determined. The relatively high concentration of BOFA in placenta and in cord serum at the end of gestation as well as its persistence in normal adult serum make it unlikely that it is immunogenic in cancer patients; on the other hand, one can hypothesise that BOFA may exert an immunosuppressive effect, protecting the foetus or the tumour cells from immunological rejection. This role has been attributed to several oncofoetal proteins, including α -2H (ref. 30), AFP³¹ and the compound recently identified by Fauve *et al.*³² in murine teratocarcinomas and in mouse trophoblast.

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Corticosteroids inhibit prostaglandin production by rheumatoid synovia

PROSTAGLANDIN synthesis is inhibited by a wide variety of non-steroid anti-inflammatory drugs, and it has been suggested that the pharmacological effects of these drugs may be attributed to their inhibition of prostaglandin (PG) biosynthesis^{1,2}. Although corticosteroids are among the most potent anti-inflammatory agents available, they lack inhibitory effects on PG synthesis in many systems^{3–6}. We have investigated the effects of several anti-inflammatory drugs on PG production by rheumatoid synovial tissue *in vitro*. Non-steroidal agents inhibit PG synthesis by the synovia and their order of effectiveness is similar to that seen in other tissues⁷. Adrenocorticosteroids are also potent inhibitors of PG production by rheumatoid synovia, and the results of these experiments are reported here.

Explants of rheumatoid synovial tissue were prepared for culture from surgical specimens as described previously⁸, and PG concentrations in the culture media were measured by radioimmunoassay. Rheumatoid synovial cultures produce mainly prostaglandins E₂ and F_{2 α} (ref. 8), therefore the PGs were assayed by antisera to PGE₂ and PGF_{2 α} and the concentrations were calculated in equivalents of PGE₂ and PGF_{2 α} , respectively. In one experiment, the PG metabolites, 15-keto-PGF_{2 α} , 13,14-dihydro-15-keto-PGF_{2 α} and 13,14-dihydro-15-keto-PGE₂ were measured using antisera of the desired specificities^{9,10}.

Table 1 Inhibition of PGE₂ production by rheumatoid synovial organ cultures by dexamethasone

Tissue R-66	Prostaglandin E ₂ (μ g ml ⁻¹) [*]	
	Day 3†	Day 6†
Control‡	6.80±0.40	4.08±0.50
Dexamethasone 10 ⁻⁴ M	0.27±0.027	<0.12
Dexamethasone 10 ⁻⁶ M	0.42±0.077	<0.12
Dexamethasone 10 ⁻⁸ M	2.16±0.31	0.133±0.008
Dexamethasone 10 ⁻¹⁰ M	6.38±0.26	4.13±0.57

^{*}PGE₂ concentrations were measured by radioimmunoassay, and each value is the mean of four determinations $\pm$ s.e.

†Medium from day 3 had been incubated with synovial tissue for the first 72 h of culture. After complete medium change, incubation was carried out for an additional 72 h and removed on day 6 for analysis.

‡Each group of four cultures was carried out in the same manner except for addition of dexamethasone at the concentrations indicated, or no additions to control cultures.

Table 2 Inhibition of PG production by rheumatoid synovial organ cultures by dexamethasone and hydrocortisone

Tissue R-67, Day 4	PGE ₂	PGF _{2α}	13,14-dihydro-15-keto-PGE ₂	15-keto-PGF _{2α}
			(μg ml ⁻¹)	
Control	8.5 ± 1.1	1.65 ± 0.29	1.94 ± 0.33	0.264 ± 0.022
Hydrocortisone 10 ⁻⁵ M	0.38 ± 0.14	0.066 ± 0.014	0.42 ± 0.08	0.066 ± 0.013
Dexamethasone 10 ⁻⁶ M	0.24 ± 0.11	0.035 ± 0.009	0.34 ± 0.11	0.050 ± 0.009

*0.009 μg ml⁻¹ of 15-keto-PGF_{2α} was present in the culture media of the control tissues and none could be found in the corticosteroid-treated cultures. The 15-hydroxyprostaglandin dehydrogenase activity in the synovia therefore seems to limit the amount of 13,14-dihydro-15-keto compounds formed from Δ¹³-reductase activity.

The production of PGE₂ and its inhibition by dexamethasone are shown in Table 1. Groups of four synovial cultures were incubated with four different concentrations of dexamethasone and compared with a control group without added drug. Concentrations of PGE₂ were reduced to <6% of the controls at both 10⁻⁴ and 10⁻⁶ M dexamethasone. Partial reduction of PGE₂ was seen at 10⁻⁸ M and there was no effect at 10⁻¹⁰ M. Slightly greater inhibition was observed in this and other experiments after the second incubation period on day 6 than on day 3, but the results were otherwise similar.

In another experiment, using a different synovial tissue, culture media were analysed for PGE₂, PGF_{2α} and three of their metabolites, the 13,14-dihydro-15-keto derivatives of PGE₂ and PGF_{2α} and the 15-keto derivative of PGF_{2α} (Table 2). In the presence of both hydrocortisone at 10⁻⁵ M and dexamethasone at 10⁻⁶ M, concentrations of PGE₂ and PGF_{2α} in culture media were reduced to <5% of untreated (control) cultures. Levels of the PG metabolites were similarly reduced, although to a somewhat lesser extent. The presence of these metabolites in culture media indicates significant activities of 15-hydroxyprostaglandin dehydrogenase and Δ¹³-reductase in rheumatoid synovial tissue. The reduced concentrations of the PG metabolites is more consistent with an inhibitory effect of corticosteroids on PG synthesis than with an enhancement of their breakdown. The observation that PGE₂ and PGF_{2α} (and their respective metabolites) are reduced by similar factors suggests that corticosteroids inhibit PG synthesis at a stage before the formation of the endoperoxide intermediates, PGG₂ and PGH₂, since PGE₂ and PGF_{2α} are formed from the endoperoxide intermediates by separate pathways¹¹.

To investigate further whether the effect of corticosteroids on PG levels in synovial cultures is the result of either inhibition of synthesis or an enhancement of degradation, we examined the stability of PGE₂ in the experimental conditions. PGE₂ (1.0 μg ml⁻¹) was added to groups of cultures treated with dexamethasone and hydrocortisone at the beginning of each 72-h incubation period, and the recovery of PGE₂ was determined by comparison of PGE₂ concentrations in each pair of cultures (Table 3). The recovery ranged from 57% to 162% of PGE₂ added, which indicates that there is no significant degradation of PGE₂ in treated cultures. In a separate experiment, it was found that PGE₂ was also stable in untreated

rheumatoid synovial cultures. These results show that the suppression of PGE₂ levels by corticosteroids cannot be accounted for by an increased rate of degradation in the culture media.

The data do not entirely exclude the possibility that corticosteroids could enhance PG degradation within synovial tissue, before the transport of PGs into culture media. This possibility seems unlikely, however, because of the reduced rather than increased levels of prostaglandin metabolites in cultures treated with corticosteroids.

Synovial tissue from the experiment reported in Table 1 was analysed for PGE₂ content at its conclusion. The results shown in Table 4 reveal that the PGE₂ content of the tissue is reduced by dexamethasone in parallel with the reduction in PGE₂ concentrations in the culture medium. The quantities of PGE₂ in untreated tissues were much lower than in culture media. For example, the culture media from the four control cultures contain over 32 μg PGE₂ at the end of the experiment on day 6 when the tissue was analysed (Table 1); in contrast, the pooled tissue from control cultures contained 0.111 μg, or <1% of that present in the medium. The results of the tissue analyses rule out the possibility that reduced PGE₂ concentrations in culture media produced by corticosteroids result solely from inhibition of transport out of the synovial tissue. It has been concluded by others¹² that corticosteroids may inhibit PG transport from adipose tissue.

Table 4 PGE₂ content of rheumatoid synovial tissue (R-66)

	μg PGE ₂	Pooled tissue wet weight (mg)
Control	0.111	147
Dexamethasone 10 ⁻⁴ M	0.017	147
Dexamethasone 10 ⁻⁶ M	0.019	125
Dexamethasone 10 ⁻⁸ M	0.026	152
Dexamethasone 10 ⁻¹⁰ M	0.065	151

Synovial tissue from all cultures R-66 (see Table 1) were pooled from the four cultures comprising each group. The tissue was removed, rinsed and blotted dry and weighed at the conclusion of the experiment, after 6 d of incubation. The tissue was homogenised at 0 °C and the homogenate was extracted with diethyl ether after acidification to pH 3 (ref. 15).

Finally, in an experiment not shown here, prednisolone at 10⁻⁶ and 10⁻⁸ M, suppressed PGE₂ levels to 2% and 42% of control values respectively, after 72 h of incubation. Therefore similar inhibitory effects have been observed with hydrocortisone and two synthetic anti-inflammatory corticosteroids.

We conclude tentatively that corticosteroids inhibit the biosynthesis of PGs by rheumatoid synovial tissue, in the absence of any detectable increase in either degradation or storage of PGs in tissue explants. The mechanism of this inhibition remains unclear and is under investigation.

It is possible that corticosteroids inhibit PG production by a nonspecific toxic effect on cells in rheumatoid synovial tissue, but evidence against this is provided by histological sections of

Table 3 Corticosteroid inhibition of PGE₂ production by rheumatoid synovia and the absence of increased PGE₂ degradation

Tissue R-70	Prostaglandin E ₂ Day 3	(μg ml ⁻¹) Day 6
Control	5.4 ± 0.67	1.98 ± 0.29
Dexamethasone 10 ⁻⁶ M	0.19 ± 0.05	0.008 ± 0.002
Dexamethasone 10 ⁻⁶ M + PGE ₂ 1 μg ml ⁻¹	1.2 ± 0.10	0.58 ± 0.12
Hydrocortisone 10 ⁻⁵ M	0.33 ± 0.06	0.017 ± 0.005
Hydrocortisone 10 ⁻⁵ M + PGE ₂ 1 μg ml ⁻¹	1.95 ± 0.11	1.21 ± 0.23

See legend to Table 1. PGE₂ was added to one of two groups of dexamethasone- and hydrocortisone-treated cultures, to determine the stability of PGE₂ in these conditions.

tissue from one experiment (Table 2) after 6 d in culture. Routine haematoxylin and eosin-stained preparations revealed no apparent differences between control and dexamethasone (10^{-6} M) treated tissues, and no histological evidence of cell necrosis was observed in either case.

The inability of corticosteroids to inhibit PG synthesis by various tissues, as intact cell preparations^{4,5}, tissue homogenates³ or isolated microsomes⁶, has been reported. This is in contrast to the sensitivity of PG synthesis by these same tissues and many others to inhibition by a variety of non-steroid anti-inflammatory drugs such as indomethacin and aspirin. In fact, we are unaware of any reports of inhibition of PG production by corticosteroids at the low concentrations used in our experiments and in those reported by Tashjian *et al.*¹³. Inhibition of PG synthesis by homogenates of human skin¹⁴ and sheep seminal vesicles¹⁵ by the steroid flucinolone, has been reported, but in both cases concentrations of the drug exceeding 0.1 mM were used and resulted in only approximately 50% inhibition of PG synthesis. In addition, hydrocortisone had no effect on PG synthesis by the skin homogenates at a concentration of 0.28 mM¹⁴. Lewis and Piper have reported that corticosteroids inhibited PG-mediated vasodilatation in a perfused rabbit adipose tissue preparation¹², and they suggested that corticosteroids inhibited the transport of PG from the interior of adipose cells to the extracellular space. Hydrocortisone seemed to cause no significant change in PGE₂ content of adipose tissue, however, and it was not possible to measure PGE₂ directly by bioassay in effluent venous blood from either the control or treated preparations. Corticosteroids were shown to inhibit the ACTH-induced vasodilatation, which has been attributed to release of PGE₂ by adipose tissue, but Lewis and Piper presented no direct evidence that corticosteroids effected either PG synthesis or release.

In summary, corticosteroids have been shown to suppress the accumulation of PGE₂ and PGF_{2 α} in culture media from explants of rheumatoid synovia and the evidence indicates that corticosteroids inhibit prostaglandin biosynthesis by this tissue.

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Hydrocortisone inhibits prostaglandin production by mouse fibrosarcoma cells

A VARIETY of non-steroid anti-inflammatory drugs are potent inhibitors of prostaglandin biosynthesis¹. In fact, many of the clinically important anti-inflammatory actions of agents such as aspirin and indomethacin have been attributed to inhibition of prostaglandin synthetase². Corticosteroids with anti-inflammatory activity, however, have not been regularly shown to be prostaglandin synthesis inhibitors^{1,3,4} although reports of small degrees of inhibition have been made in broken cell preparations using very high concentrations (2.2×10^{-4} – 4.4×10^{-4} M) of flucinolone^{5,6} or hydrocortisone⁶. Lewis and Piper have suggested, largely on the basis of indirect data, that some of the actions of corticosteroids in inflammation result from inhibition of the release, but not the synthesis, of prostaglandins⁷. We report here that hydrocortisone is a potent inhibitor of prostaglandin formation by a clonal strain of prostaglandin-producing cells in culture, and that the effect is not the result of an action on the prostaglandin release or transport mechanisms.

The cells used were the HSDM₁C₁ strain of mouse fibrosarcoma cells which synthesise and secrete substantial quantities of prostaglandin E₂ (PGE₂)^{8,9}. Cell culture methods have been described⁹ (see also legend to Fig. 1).

Addition of hydrocortisone to cultures of HSDM₁C₁ cells

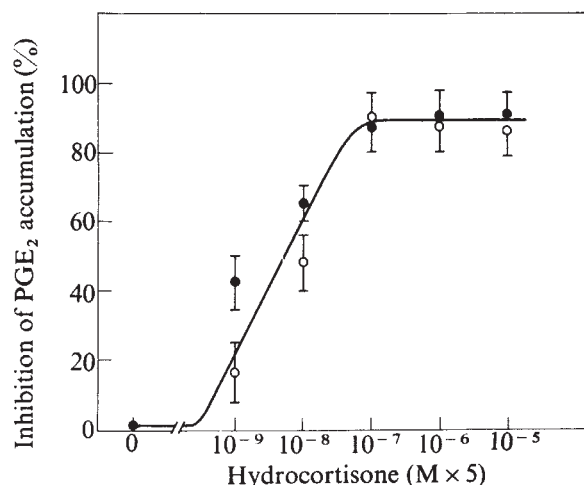


Fig. 1 Inhibition of PGE₂ accumulation in the medium of HSDM₁C₁ cultures produced by hydrocortisone. Cells were grown in Ham's F10 medium supplemented with 15% horse serum and 2.5% foetal calf serum at 37 °C in a humidified atmosphere of 5% CO₂ in air. Total cell protein was determined by the method of Lowry *et al.*¹⁰ at the time of each medium collection. PGs^{11,12} and PG metabolites^{13,14} in unextracted medium were measured by serological techniques. We have shown that PGE₂ is the predominant prostaglandin synthesised and secreted by HSDM₁C₁ cells^{8,9}. Media were usually assayed at final dilutions of 1/200 to 1/20,000, where there are no nonspecific effects of medium on the assay methods used. For measurements of intracellular PGs, the cells were homogenised in acidified (pH 3.5) Gey's balanced salt solution (about 2 ml mg⁻¹ cell protein) containing indomethacin (1 µg ml⁻¹). PGs in the aqueous extract were then concentrated by extraction into diethyl ether as previously described⁹. Most experiments were performed using hydrocortisone sodium succinate (Solu-Cortef, Upjohn) although the same results were obtained with hydrocortisone. Each molar value read on the abscissa multiplied by 5 gives the final concentration of hydrocortisone in medium. Each point is the mean of duplicate culture dishes and the bars the ranges. Results are for culture periods of 4 (●) and 7 (○) d, respectively. After 4 d of incubation with or without hydrocortisone, medium was collected for assay and fresh control or hydrocortisone-containing medium was added for an additional 3-d period. In control cultures, PGE₂ accumulation averaged 150 ng ml⁻¹ medium per 24 h.

produced a dose-dependent decrease in the accumulation of PGE_2 in the medium (Fig. 1). The half-maximum effect was observed at about 1×10^{-9} M hydrocortisone, and the extent of inhibition was 90–95% complete at maximum effective concentrations (5×10^{-7} to 5×10^{-6} M). In long term experiments (≥ 4 d), hydrocortisone concentrations of 10^{-7} M or lower had no significant effects on the rate of cell growth.

The time course and reversibility of the hydrocortisone effect are shown in Fig. 2. Inhibition of PGE_2 accumulation in medium was marked after 8 h incubation with the steroid hormone, and little or no further accumulation of PGE_2 occurred in the continued presence of 5×10^{-6} M hydrocortisone in spite of an increasing accumulation in controls. In cultures in which the drug was removed at the end of the 8-h treatment, there was a rapid return of PGE_2 accumulation in medium at a rate comparable with that in controls, indicating that the inhibition was not an irreversible toxic effect on the cells. Similar reversibility was observed in cultures treated with hydrocortisone for 24–72 h (data not shown). Other experiments showed that the inhibitory effect of the corticosteroid could be detected as early as 4 h after its addition to the culture medium.

Lewis and Piper stated that confirmation of their hypothesis of inhibition of prostaglandin release by corticosteroids might be accomplished by experiments with isolated cells⁷. We have, therefore, measured both the extracellular secreted PGE_2 and intracellular stored PGE_2 in control, hydrocortisone-, and indomethacin-treated cultures. If the steroid hormone were acting merely to inhibit the release of PGE_2 , PG should accumulate intracellularly and exceed the amount found in control cells. Figure 3 shows that this is not what is found. Intracellular PGE_2 was not elevated in cells treated with hydrocortisone for 48 h although there was marked inhibition of PGE_2 accumulation extracellularly. Indomethacin, known to be a potent inhibitor of PGE_2 synthesis in HSDM₁C₁ cells^{8,9,15}, produced essentially complete inhibition of PGE_2 accumulation in medium and depressed intracellular PGE_2 to about 40% of control values (Fig. 3a). Removal of both hydrocortisone and indomethacin at 48 h was followed by a return of PG accumulation in the medium to control, or even elevated levels by an additional 24–48 h, again indicating that neither drug produced irreversible toxic effects on the cells.

Fig. 2 Time course and reversibility of the effect of hydrocortisone (5×10^{-6} M) on PGE_2 accumulation in the medium of HSDM₁C₁ cultures. Medium lacking ($\circ$) or containing hydrocortisone ($\bullet$ and $\blacktriangle$) was added at zero time to groups of replicate cultures. Duplicate dishes were taken and medium collected at each of the times shown for PG assay. *a*, Control cells without hydrocortisone; *b*, cultures received hydrocortisone continuously throughout; *c*, cultures received HC for the first 8 h only and control medium thereafter. There were no differences in total cell protein between groups throughout the experiment, averaging 0.80 mg per dish at zero time and 1.3 mg per dish at 38 h. Each symbol gives the mean value of duplicate dishes and the bars the ranges.

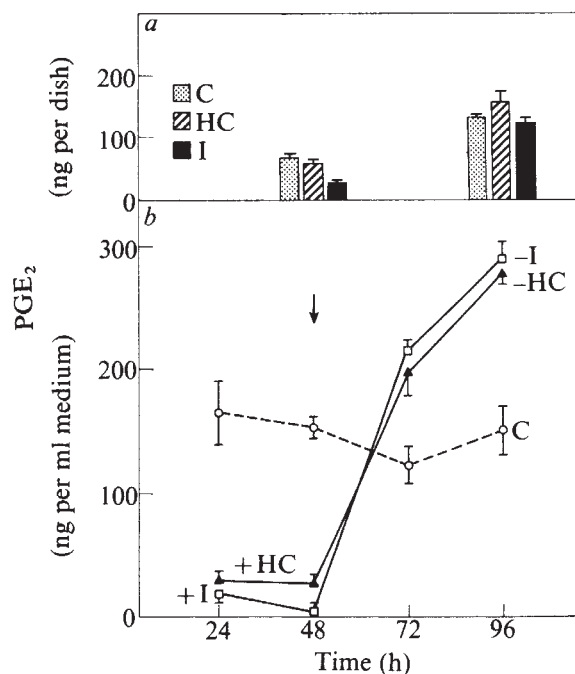
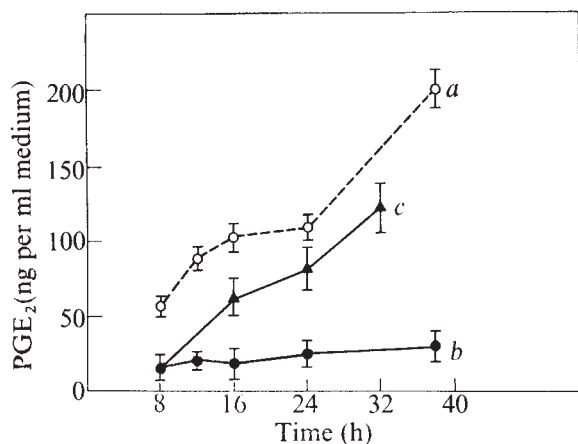


Fig. 3 Effects of hydrocortisone and indomethacin on intracellular (*a*) and extracellular (*b*) PGE_2 in HSDM₁C₁ cultures. Replicate dishes were cultured without ($\circ$) drug or with hydrocortisone ($\blacktriangle$, 5×10^{-6} M) or indomethacin ($\square$, 5.6×10^{-7} M) for 48 h. At that time (arrow), fresh medium lacking hydrocortisone or indomethacin was added to the previously treated dishes and incubations continued for an additional 48 h. Medium from all dishes was collected for assay and appropriate fresh medium added every 24 h. Each symbol gives the mean of duplicate dishes and the bars the ranges. *a*, Data for intracellular PGE_2 in control (C) and hydrocortisone (HC)- and indomethacin (I)-treated cultures at 48 and 96 h. Each block represents the mean of duplicate dishes and the bars the ranges. In three additional experiments intracellular PGE_2 content in HC-treated cultures was 74, 82 and 68% of control.

We conclude that in the HSDM₁C₁ cell culture system hydrocortisone is a potent inhibitor of the accumulation of PGE_2 in the extracellular medium. The effect cannot be explained by a simple inhibition of PGE_2 release or transport⁷ because there is no concomitant increase in intracellular PGE_2 , in fact, a decrease in intracellular PGE_2 was usually observed. We do not believe that hydrocortisone has a nonspecific, toxic effect on cell metabolism because its actions occur at low steroid concentration, are reversible, occur rapidly, and are not accompanied by a decrease in the growth rate of the cells. It could be argued that hydrocortisone acts to cause the observed effect by accelerating the degradation of PGE_2 or by altering the ratios of PGE_2 to $\text{PGF}_{1\alpha}$ or $\text{PGF}_{2\alpha}$. We do not believe that enhanced breakdown of PGE_2 is the explanation, for we cannot detect the metabolites 13,14-dihydro-15-keto- PGE_2 , 13,14-dihydro-15-keto- $\text{PGF}_{2\alpha}$ or 15-keto- $\text{PGF}_{2\alpha}$ in hydrocortisone-treated cultures (0.5 ng ml^{-1} of these metabolites could have been measured in control and hydrocortisone-treated culture media by our radioimmunoassay techniques). In addition, exogenous PGE_2 is not degraded by cells previously treated with hydrocortisone. PGE_2 (51 ng ml^{-1}) was added to cultures treated with hydrocortisone (5×10^{-6} M) for 24 h. Control hydrocortisone-treated cultures received no added PGE_2 . In a subsequent 5-h incubation (from 24–29 h), $99 \pm 3\%$ (mean $\pm$ s.e.) of the added PGE_2 was recovered. When culture medium was assayed for $\text{PGF}_{2\alpha}$ as well as PGE_2 , no changes in the ratios of PGE_2 to $\text{PGF}_{2\alpha}$ were found; the ratios of PGE_2 to $\text{PGF}_{2\alpha}$ in one experiment were 31 and 26 in control and hydrocortisone-treated cultures respectively, and in a second experiment, 34 and 32, respectively.

We propose therefore that in intact HSDM₁C₁ cells, hydrocortisone inhibits PGE_2 accumulation in medium by inter-

fering with PG synthesis. Although the locus of this effect remains unknown at present, the unaltered ratios of PGE_2 to $\text{PGF}_{2\alpha}$ indicate that the block in synthesis precedes endoperoxide formation. The simplest explanation at the moment is that the corticosteroid binds to membrane structures and prevents activation of the prostaglandin synthesis system of the intact cell. The similarity of our findings to those of Kantrowitz *et al.*¹⁶ in a different experimental system, lead us to believe that these effects may be a more general action of corticosteroids and may be important in their activity as anti-inflammatory agents.

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Spontaneous calcium action potentials in a clonal pituitary cell line and their relationship to prolactin secretion

It has been suggested that in the adrenal medulla calcium ions enter the cell as a result of depolarisation caused by acetylcholine¹. During this process it is conceivable that a mechanism of membrane potential-dependent calcium permeability change, that is, the calcium action potential, is involved. A certain amount of calcium ions may enter the cell during an action potential and the modulation of the action potential frequency may provide a sensitive way to control the calcium entry. Similar mechanisms may be involved in other secretory systems. Here I describe an attempt to prove this hypothesis in anterior pituitary cells.

A clonal cell line (GH₃) isolated from a rat anterior pituitary tumour has been shown to secrete continuously both growth hormone and prolactin^{2,3}. A hypothalamic releasing factor (thyrotropin releasing factor TRF) stimulates prolactin secretion and inhibits growth hormone secretion at nanomolar concentrations⁴.

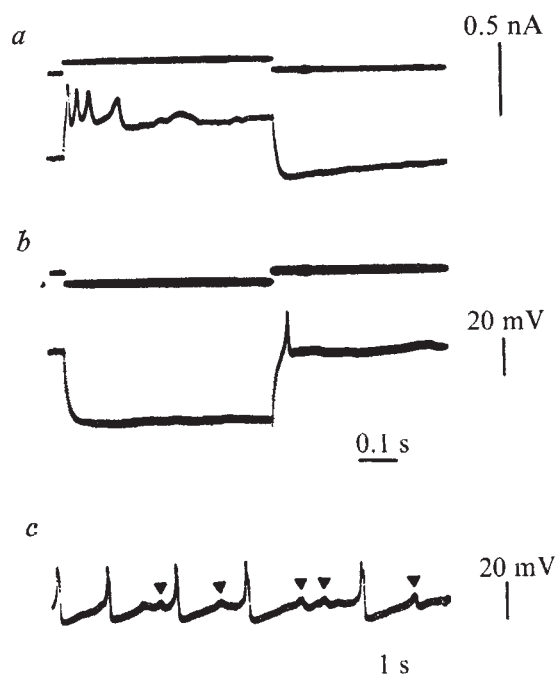
Electrical membrane properties of these cells were studied using intra- and extracellular microelectrode recording techniques, and their correlation with secretory activities is discussed. Cells were grown in Ham's F10 medium supplemented with 15% horse serum and 2.5% foetal calf serum in an atmosphere of 5% CO₂ in air. Intracellular recording and current injection

using a bridge circuit were carried out as described previously⁵ in a saline of the following composition: NaCl 142.6 mM, KCl 5.6 mM, CaCl₂ 10 mM buffered by 5 mM HEPES–NaOH to pH 7.4. A high calcium concentration in this saline seemed to enable good microelectrode penetration of the cell. A suction microelectrode with an inner tip diameter of less than 5 μm was used for the extracellular recording. A small part of the cell surface membrane was slowly sucked into the electrode (about 5–10 μm) making a good electrical contact. The action potentials recorded in this way usually have a positive-negative deflection of 0.1–1 mV peak-to-peak amplitude (Fig. 2a). For extracellular recordings a saline of the following composition was used: NaCl 152 mM, KCl 5.6 mM, CaCl₂ 1.8 mM, sucrose 38 mM, buffered by 5 mM HEPES–NaOH to pH 7.4. Sucrose was added to adjust the tonicity of a saline to that of culture medium. All experiments were carried out at room temperature (19–26 °C). The frequency of spontaneous action potentials is affected by the mechanical deformation caused by the suction electrode. To avoid this artefact as much as possible, action potential frequency was estimated only after the spontaneous firing rate had been stable for more than 5 min with little or no change in the configuration of the recorded action potential.

Stable intracellular recordings of these cells were difficult, probably because the cells were small (10–20 μm diameter when spherical). Relatively stable recordings were occasionally obtained in cells attached to the bottom of culture dishes. The mean resting membrane potential in these cells was -41 ± 4 mV (mean $\pm$ s.d., $n = 8$), which is more negative than that (-12.1 mV) recorded in adenohypophyseal cells in tissue culture⁶. The resting membrane potential obtained in this experiment may, however, be an underestimate of the real value. The input resistances of these cells measured by applying small hyperpolarising currents were 340–700 M Ω , which is larger than the value (6.3 M Ω) reported by York⁶. In all successfully penetrated cells, a prominent fluctuation of the membrane potential of about 4 mV amplitude was observed (Fig. 1c, triangles). These fluctuations occasionally initiated action potentials. Similar fluctuation of the membrane potential has been observed in pancreatic β cells⁷.

Action potentials could be evoked after termination of a hyperpolarising current (Fig. 1b) or several action potentials

Fig. 1 Intracellular recordings of a GH₃ cell. Upper line in each record indicates zero membrane potential level as well as current (in a and b). Lower traces are membrane potentials. c, Spontaneous action potentials shown. ∇ , Small membrane potential deflections.



were observed with each depolarising pulse (Fig. 1a). The action potentials were followed by an after-spike hyperpolarisation, and had several characteristics in common with a calcium action potential⁸. Thus they were not blocked by tetrodotoxin (2×10^{-6} M) nor by substituting equiosmolar Tris-HCl for the NaCl and HEPES-NaOH, but could be abolished by adding LaCl_3 (1 mM) or MnCl_2 (5 mM) to the medium. Therefore it is likely that these action potentials were due to a membrane conductance increase to calcium ions.

Spontaneous action potentials were recorded extracellularly. All cells showed spontaneous action potentials at random intervals with a frequency of $52 \pm 20 \text{ min}^{-1}$ ($n = 20$) at room temperature (Fig. 2b). The frequency of action potentials in any given cell was stable (coefficient of variation: 15 in average of 6 cells) although the frequency varied from cell to cell (coefficient of variation: 38). After TRF acetate was added to the medium, action potential frequency was measured in randomly chosen cells. The mean frequency in the presence of 30 nM TRF was $91 \pm 32 \text{ min}^{-1}$ ($n = 13$) which is significantly larger than the control ($P < 0.001$), whereas with 1 nM TRF the mean frequency was $52 \pm 26 \text{ min}^{-1}$ ($n = 6$) which is not different from the control. Moreover, the spike frequency after addition of 231 nM [πMeHis^2]-TRF (inactive TRF derivative)^{4,9} was $60 \pm 26 \text{ min}^{-1}$ ($n = 9$), which is not different from the control.

In this system^{10,11} as well as in many others¹² extracellular calcium ions have been shown to be required for hormone secretion. The level of prolactin secretion of the anterior pituitary cells used in this experiment is reported to increase immediately after addition of TRF (0.3 μM) to several times the basal level⁴. Similarly the frequency of spontaneous calcium action potentials increased to about two times the control after adding a small amount of TRF. [πMeHis^2]-TRF did not affect the action potential frequency or the hormone secretion^{4,9}.

The correlation between hormone secretion and calcium action potentials in this system in various conditions suggests that calcium ions traversing the membrane during action potentials may be related to the hormone secretion, as has been suggested in pancreatic β cells¹³.

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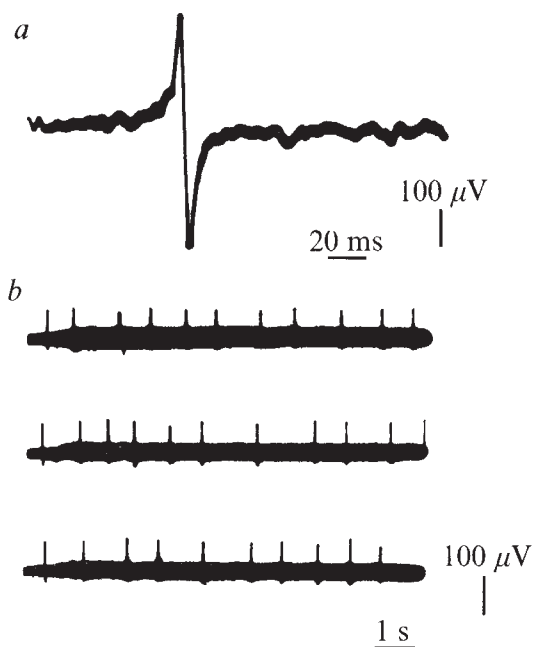
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Effect of oestrogen antagonist on hypothalamic oestrogen receptors

THE oestrogen antagonist CI-628 can block completely oestrogen-induced sexual receptivity in rats even when the antagonist is given as long as 6 h after oestrogen¹. But as CI-628 does not displace labelled oestradiol from hypothalamic tissue when given at times when it does block the behavioural effects of the oestrogen, the inhibition is not due to simple competitive inhibition. The behavioural inhibition, however, might be due to an inhibition of the action of oestrogen on hypothalamic receptors. This was suggested by the report that oestrogen antagonists can deplete cytoplasmic oestrogen receptors in the uterus², but fail to stimulate production of new receptor, a process which is induced by oestrogen. We have now shown that CI-628 depletes hypothalamic oestrogen receptors, that receptor replenishment occurs very slowly and that CI-628 delays receptor replenishment after oestradiol treatment, suggesting that receptor replenishment might be important for the behavioural effects of oestradiol.

Eighty female rats were ovariectomised at 8 weeks of age and used 4-25 d later. Groups of five rats were injected intravenously with 2.5 μg of oestradiol, 500 μg of CI-628 (1-[2-(p-[α -(p-methoxyphenyl)- β -nitrostyryl] phenoxy) ethyl] pyrrolidine monocitrate) (Parke-Davis) or with oestradiol followed by CI-628; control animals were not injected. The animals were killed 1-24 h after injection. The hypothalamus was removed, homogenised in TE buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.4) and centrifuged for 1 h at 105,000g to yield a high speed supernatant (cytosol) fraction. Twenty-five microlitres of 2,4,6,7-³H-oestradiol (specific activity 104.8 Ci mmol⁻¹, New England Nuclear) in TE buffer was added to 0.1 ml of each cytosol to give a concentration of 1×10^{-9} M. Further 0.1-ml aliquots were treated with ³H-oestradiol plus a 100-fold excess of unlabelled oestradiol. The samples were incubated overnight at 4 °C. A third aliquot was taken for protein determination using the Miller modification³ of the Lowry procedure. Protein concentrations were between 8 and 10 mg ml⁻¹. Cytoplasmic oestradiol binding was assayed using a Sephadex G-25 minicolumn technique⁴. Specific binding was determined from the difference in binding between the cytosols incubated with ³H-oestradiol and cytosols treated with ³H-oestradiol plus a 100-fold excess of unlabelled oestradiol. Binding was determined as d.p.m. per mg protein and is expressed as percentage of control. The control receptor levels varied in different experiments with a mean of 552.4 d.p.m. per mg protein. This technique

Fig. 2 Extracellular recordings of spontaneous action potentials. Upward deflection indicates positive potential change at the electrode referring to the bath. a, Fast sweep record; b, spontaneous action potentials recorded with slower sweep speed. a and b are records from different cells.



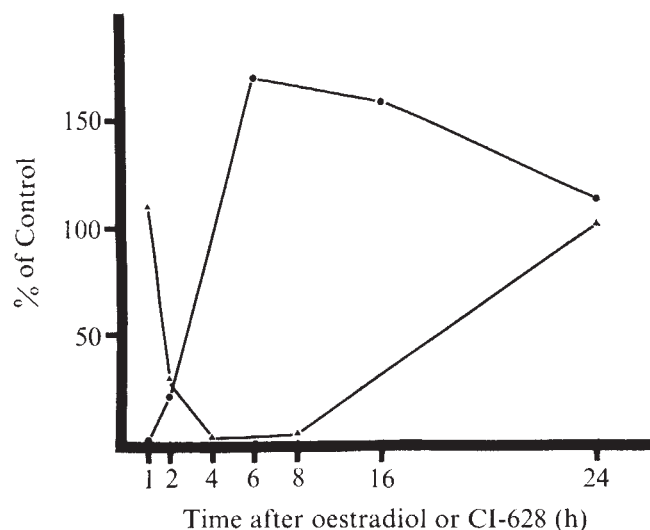


Fig. 1 Relative levels of saturable binding in cytoplasm from hypothalamic tissue of ovariectomised rats at various times after intravenous treatment with oestradiol (●) or the anti-oestrogen CI-628 (▲).

measures only unfilled receptor sites and thus may give an underestimate of total sites.

Figure 1 shows the changes which occur in the level of hypothalamic oestrogen receptors after intravenous treatment with either oestradiol or CI-628. One hour after oestradiol treatment the receptors were completely depleted; partial replenishment was seen after 2 h, and at 6 h receptor levels were more than 150% of control values. Levels returned to control values by 24 h after oestrogen treatment. The pattern of change after CI-628 treatment was different from that produced by oestrogen. Receptor depletion was slower, reaching a minimum value 4 h after treatment; replenishment was slower and, for the dose used and at the times of measurement, no overshoot was observed. Others have reported that CI-628 induced receptor depletion, but found no receptor replenishment⁵ as observed in the study reported here.

Table 1 shows the effects of CI-628 administered at various times after oestradiol treatment on receptor levels 24 h after oestrogen had been given. Receptor levels were quite low in each case. Comparison of these data with those in Fig. 1 suggests that CI-628 prevents, at least at the times examined, replenishment of receptors when given shortly after oestradiol and depletes receptors which are generated by long term (8 or 16 h) oestrogen stimulation.

Our data suggest that the inhibitory effects of CI-628 on sexual activity are due to an inhibition of receptor replenishment. But the action of CI-628 may be more complex. It has been shown that the oestradiol can be translocated to the nucleus of uterine cells after treatment with anti-oestrogen but that it has little effect on uterine growth⁶. Thus anti-oestrogens can both block cytoplasmic receptor replenishment and interfere with interactions of oestrogen-receptor complexes with the nucleus.

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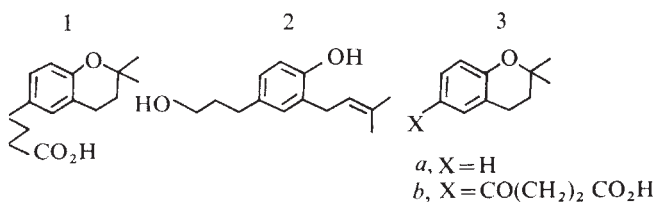
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New antisickling agent 3,4-dihydro-2,2-dimethyl-2H-1 benzopyran-6-butyric acid

RECENT efforts to develop therapeutic agents for the treatment of sickle cell disease have led to the discovery of several anti-sickling agents¹. We now report a new such agent, 3,4-dihydro-2,2-dimethyl-2H-1-benzopyran-6-butyric acid (DBA) (Compound 1). This compound efficiently prevents and reverses sickling, and has no acute toxicity in male mice.



Fagara xanthoxyloides Lam, a small tree with shining aromatic leaves is common in coastal areas of West Africa. The roots, which have a strong spicy taste, are used in Nigeria as chewing sticks which are chewed to a brush and used to clean the teeth. Various natural products^{2,3} have been isolated from the plant, and the crude aqueous extract of the roots has been investigated for its antimicrobial⁴ and antisickling⁵ activity. Root bark and root wood extracts gave, in addition to other natural products (unpublished results of J.I.O., D.E.U.E. and V.U.E.), xanthoxylol (compound 2). In preliminary bioassays on xanthoxylol (carried out by Dr A. Isaacs-Sodeye, University College Hospital, Ibadan) it seemed to possess antisickling activity. We have chemically modified xanthoxylol to DBA, and have found that the latter is a very active antisickling agent. It has also been synthesised efficiently, in 90% overall yield, by Friedel Crafts acylation of 2,2-dimethylchroman (3a) (obtained from dihydrocoumarin) with succinic anhydride, followed by Clemmensen reduction of the keto-acid (3b). ¹⁴C-DBA was prepared using 1,4-¹⁴C-succinic anhydride in the acylation step.

Sickling was produced by one of the following methods: (1) reduction with 2% metabisulphite; (2) evacuation at 30 mmHg with a water aspirator for 10 min at 37 °C; and (3) bubbling a mixture of 95% N₂ and 5% CO₂ through a saline suspension of sickle cells for periods of 10 min to 2 h at 37 °C. In all cases about 90% sickling was attained.

Hanging drop preparations (10 µl) of deoxygenated cell suspension were maintained in the deoxygenated state and treated with various amounts of DBA. One thousand cells were counted at suitable intervals by light microscopy (Zeiss, ×600). As Table 1 shows, the effect of DBA was already apparent after 30 min, the minimum effective concentration being 0.9 mM. In addition, the cell suspensions were placed in 10-ml Erlenmeyer flasks arranged in series in a 37 °C waterbath, and

Table 1 Effect of CI-628 on hypothalamic cytoplasmic oestrogen receptor levels.

Hours after E2 when CI-628 was given	% of Control
2	25.2
4	20.4
8	1.6
16	7.5

Animals were killed 24 h after treatment with oestradiol.

Table 1 Effect of DBA on deoxygenated sickle cells at different times

Concentration (mM)	% Sickle cells	30 min % Varied shape cells	% Normal shape cells	% Sickle cells	60 min % Varied shape cells	% Normal shape cells	% Sickle cells	90 min % Varied shape cells	% Normal shape cells
Control	69	29	2	69	29	2	72	26	2
10	1	7	92	0	11	89	3	3	94
6	2	18	80	2	21	77	2	12	86
3	3	36	61	4	25	71	6	34	51
1.8	8	31	61	3	41	56	7	40	53
0.9	46	32	22	44	46	10	23	54	23

1,000 cells were counted.

deoxygenated. Various concentrations of the drug were added, the flasks were incubated for 1 h and the cells were fixed by dilution with deoxygenated, buffered formalin. The results obtained on these fixed preparations were similar to those shown in Table 1. The erythrocyte indices were not affected when sickle cells were incubated with DBA and deoxygenated.

The inhibitory effect of DBA on sickle cell formation was tested by incubating sickle cell suspensions with three concentrations of DBA for 1 h, deoxygenating the cells as described previously, and then counting the percentages of sickle and normal cells. The control sample remained oxygenated, and contained 98% (patient A) and 78% (patient B) normal shaped cells, respectively. As Table 2 shows, the antisickling effect was already observed with 1 mM DBA, and inhibition of sickling was almost complete at 13 mM. The antisickling effect with 7.5 mM DBA is shown in Fig. 1.

As Fig. 2 shows, the oxygen pressure (in mmHg) at which the test blood was half-saturated (P_{50}) did not change significantly from the control specimen, a slight shift, if any, occurring towards the right. This again is unlike most other antisickling agents, for example, cyanate⁸, dimethyl adipimidate¹⁰ and nitrogen mustard⁹, which shift the oxygen affinity curve to the left. These results indicate that there may be no interference with the delivery of oxygen to tissues.

When sickle cell anaemia haemolysates were exposed to different concentrations of DBA for 1 h gelling was inhibited at a concentration of 2 mM (Table 3).

¹⁴C-DBA incorporation studies (Table 4) showed that very little of the drug used for incubation actually enters the red cells. Of the counts incorporated most were associated with non-haemoglobin protein (trichloroacetic acid (TCA) soluble part). Carboxymethyl cellulose chromatography, at pH 6.7, of globin from the lysate of cells incubated with ¹⁴C-DBA revealed that the ¹⁴C counts remained just above background and were distributed nonspecifically throughout the column. The ¹⁴C counts were associated with neither α nor β chains. The α/β ratio as estimated from ³H counts was 1. Thus unlike other antisickling agents such as potassium cyanate which binds to the α and β chains of oxyhaemoglobin^{15,16}, DBA does not bind covalently to sickle haemoglobin. The activity of DBA thus seems to be due to non-covalent binding to haemoglobin or interaction with the red cell membrane.

The rate of potassium loss from sickle cells provides an accurate method of measuring the degree of sickling¹⁷. In a preliminary experiment, a sample of sickle blood (Hct 30%) was incubated for 30 min at 37 °C in air. Ouabain (10^{-4} M) was then added to inhibit active cation transport. The sample was

Table 2 Antisickling effect of DBA

	DBA (mmol)	% Normal cells
Patient A	3.8	87
	8.0	93
	13.0	95
Patient B	1.0	48
	6.0	64

Table 3 Inhibition of gelation by DBA

DBA concentration (mol $\times 10^{-4}$)	Hb concentration g per 100 ml
Control (without DBA)	29.7
1.98	35.0
3.92	35.7
9.52	37.6 (non-gelling Hb conc.)

A unit of blood was obtained after exchange transfusion therapy of a patient whose starch gel electrophoresis revealed HbA = 51.9 and HbS = 48.1. The haemolysate was prepared by the method of Drabkin¹¹, and was concentrated by pressure dialysis and ultrafiltration against 0.15 M potassium phosphate at pH 7.35. The lysate (0.5 ml, with and without DBA) was deoxygenated in a 10-ml Erlenmeyer flask at 25 °C on a rotary shaker under a stream of water-equilibrated N₂. Gelling inhibition studies were carried out according to the method of Bookchin *et al.*¹².

then deoxygenated (95% N₂, 5% CO₂) for 30 min. Another sample of sickle blood containing 7 mM DBA was submitted to the same treatment. The content of potassium in the plasma was determined by flame photometry (Instrument Lab. 243). This showed that red cell potassium loss induced by hypoxia was decreased by 6 meq l⁻¹ of cells in the treated sample with DBA. Experiments to observe the effect of the drug on red cell potassium loss using physiological gas mixtures for deoxygenation are in progress.

Table 4 Incubation of sickle cells with ¹⁴C-DBA

Fraction	¹⁴ C c.p.m.
(a) Total c.p.m.	about 2.40×10^6
(b) Clear supernatant	2.34×10^6
(c) Stroma	1,400
(d) Haemolysate, hot TCA insoluble	1,600
(e) TCA filtrate	25,200

Packed sickle cells (0.5 ml) were washed three times with isotonic saline and incubated at 37 °C for 1 h with pH 7.4 buffer, phosphate buffered-saline (PBS), or Krebs Ringer bicarbonate (KRB), and ¹⁴C-DBA 1.09×10^5 d.p.m. μmol^{-1} (11 mM solution, total count about 2.40×10^6 c.p.m. (fraction a)). ³H-leucine was used as an internal control to measure incorporation into globin. After incubation, the packed cells were separated from the clear supernatant buffer by centrifugation at 2,000 r.p.m. for 10 min, and the radioactivity of the supernatant was counted. The cells were then washed twice with isotonic saline until the counts in the washing were negligible, and the radioactivity of the original supernatant and combined washings was obtained (fraction b). The cells were lysed with four volumes of water, and the stroma was removed by centrifugation at 15,000 r.p.m. for 20 min at 4 °C. The stroma was washed several times with a large excess of 1 M EDTA. The ¹⁴C counts of the residual stroma was 1,400 c.p.m. (fraction c). Sample of lysate was then fractionated with a 0.45- μm millipore filter into hot TCA-soluble (non-haemoglobin) and hot TCA-insoluble (haemoglobin) protein fractions; the countings of the two fractions showed that the radioactivities of the haemoglobin protein and non-haemoglobin protein were, respectively, 1,600 and 25,200 c.p.m. (fractions d and e). Globin was precipitated from the remainder of the haemolysate by extraction with hydrochloric acid-acetone, and further purified by carboxymethyl cellulose chromatography using a modification of the method originally described by Clegg *et al.*¹³ and detailed elsewhere¹⁴. Samples were counted using a Packard Tricarb liquid scintillation counter. No significant ¹⁴C counts accompanied the α and β haemoglobin chains.

The acute toxicity of DBA was studied (at the Teijin Institute for Biomedical Research, Tokyo) by both intraperitoneal and oral administration to male mice. Intraperitoneal DBA caused sedation at 250 mg kg^{-1} ; sedation, ataxia, analgesia and myorelaxation at 500 mg kg^{-1} ; convulsion and other toxic syndromes at 750 mg kg^{-1} , and Straub's tails, chronic convulsions, depression of respiratory rate and death at $1,000 \text{ mg kg}^{-1}$. The LD_{50} was judged to be 710 mg kg^{-1} . Oral administration of DBA up

Fig. 1 Antisickling effect of 7.5 mM DBA (Zeiss, $\times 600$). Fresh heparinised blood samples collected from patients with homozygous sickle cell anaemia were kept at 4°C , and used within 6 h of venipuncture. The erythrocytes were washed three times with isotonic saline. A cell suspension was made by diluting the cells tenfold with phosphate-buffered saline at pH 7.2. *a*, Control cell suspension was incubated at 37°C in buffer for 1 h then deoxygenated for 1.5 h by method (c) (see text). *b*, Cell suspension with added 9 mM DBA was incubated similarly and deoxygenated. Cells treated with DBA had no sickle forms.

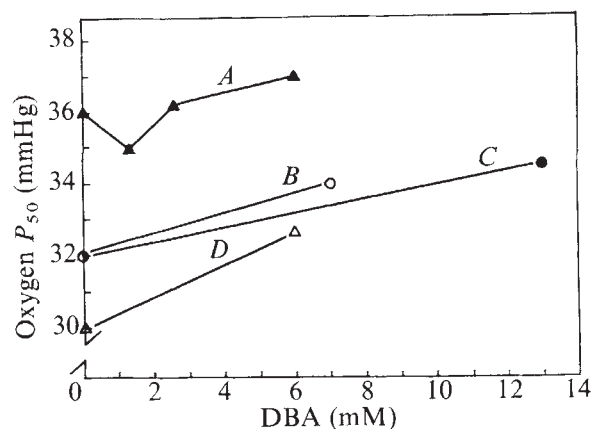
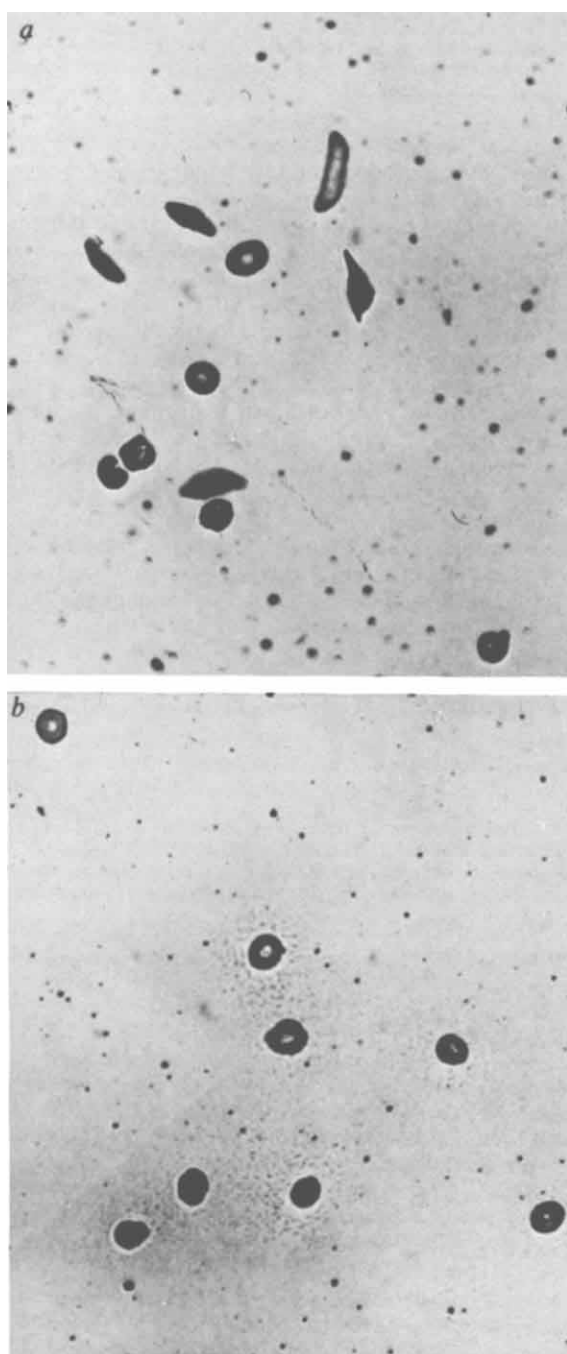


Fig. 2 Effect of DBA on the oxygen affinity of HbS. The P_{50} values from four patients (A–D) with sickle cell anaemia were determined before and after treatment with various concentrations of DBA. The P_{50} value of blood incubated with DBA was determined by the mixing technique of Edwards and Martin⁶. Whole blood (5 ml) or cell suspensions were used. The test samples were incubated with different concentrations of DBA for 1 h. Oxygen saturation was measured in a co-oximeter model 182 (Instrumentation Laboratory, Inc.). Measurements of P_{O_2} , P_{CO_2} and pH were made with a model 213 blood gas analyser (Instrumentation Laboratory, Inc.). For calculation of P_{50} , the P_{O_2} values were corrected to pH 7.40 using the Severinghaus nomogram⁷.

to $3,000 \text{ mg kg}^{-1}$ resulted in weak sedation; with doses greater than $1,000 \text{ mg kg}^{-1}$, we also observed piloerection and weak myorelaxation. The LD_{50} for oral administration was thus judged to be $> 3,000 \text{ mg kg}^{-1}$.

The effect of DBA on the smooth muscle was tested on isolated guinea pig ileum; contraction occurred with concentrations greater than $1 \times 10^{-8} \text{ g ml}^{-1}$. Finally, its effect on blood pressure was tested using the rat cardiovascular system. Intravenous injection of doses less than 1 mg kg^{-1} resulted in transitory hypertensive action (about 15–18 mmHg), whereas injection of more than 5 mg kg^{-1} produced transitory hypotensive action (about 25 mmHg).

The *in vitro* properties of DBA described here, together with the lack of acute toxicity, suggest that this compound may be a useful therapeutic agent. Further studies along these lines and on other compounds related to DBA are in progress.

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Immunohistochemical localisation of S-100 protein in brain

It is accepted that the brain-specific protein S-100 is associated predominantly with glial cells¹⁻⁵. More equivocal evidence exists to suggest a possible relationship of S-100 to neurones^{3,6,7}. Although well characterised antisera against S-100 have long been available⁸, few immunohistochemical studies of the location of S-100 antigen in brain have appeared. Two such studies have reported that S-100 is associated with neuronal elements^{9,10} but in both cases the major glial fraction of S-100 does not appear to have been localised by the immunohistochemical techniques used. The recent study by Haglid *et al.*¹⁰ claimed to demonstrate that S-100 antigen is associated with the junctional membranes of brain synapses, and on this basis an ambitious theory of chemical events underlying "learning" has been proposed¹¹ in which S-100 is assigned a key role. We have now reinvestigated the distribution of S-100 in brain tissue using a specific antiserum against S-100 (a generous gift from Dr L. Levine) which gave a positive response against rat brain extract at 1 : 1,400 dilution in complement fixation assay. The immunological properties of this antiserum have been described in detail by Kessler *et al.*¹².

Brain tissue was obtained from rats fixed by transcardiac perfusion with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4; immunofluorescence procedure) or the same fixative with the addition of 0.5% glutaraldehyde (peroxidase procedure). Cryostat sections (10 μ m) were used in immunofluorescence procedure while tissue slices were used

Fig. 1 Cryostat section of rat cerebral cortex after treatment with non-immune serum by the immunofluorescence procedure described in the text. No immunofluorescent labelling is visible. Autofluorescent granules appear in pyramidal cell bodies (arrows). Magnification, $\times 330$.

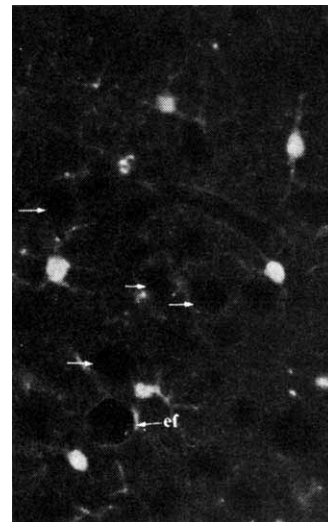
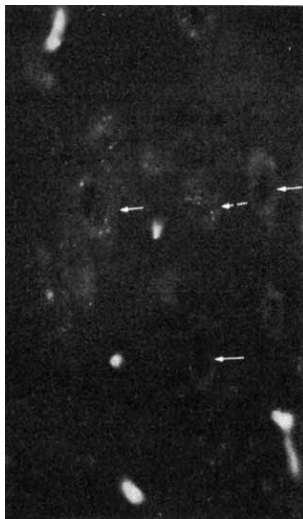
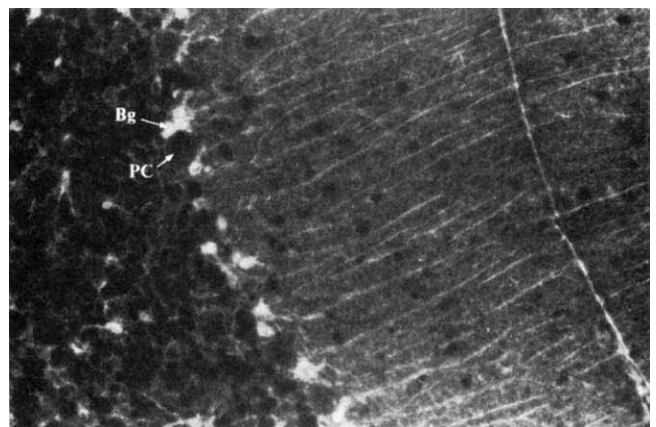


Fig. 2 Cryostat section of rat cerebral cortex treated with anti-S-100 serum for immunofluorescence. Immunofluorescent labelling is present in scattered cells whose morphology and distribution conform to that of glial astrocytes. Astroglial endfeet (ef) on blood vessels are also labelled. Fluorescence is notably absent from neuronal cell bodies (arrows). Magnification, $\times 330$.

for the immunoperoxidase procedure, cut on a Mickle-McIlwain tissue chopper set at 100 μ m. In both cases tissue samples were first incubated at room temperature with a 1 : 50 dilution of non-immune serum or one of the antisera, washed in three changes of PBS (0.15 M sodium chloride in 0.1 M phosphate buffer pH 7.4) and reincubated as before with either fluorescein-labelled sheep anti-rabbit IgG or peroxidase labelled goat and anti-rabbit IgG. After washing with PBS the peroxidase reaction was developed with 5 mg of diaminobenzidine in 10 ml of PBS containing 1 μ l of 30% hydrogen peroxide. The electron microscope samples were postfixed in 1% osmium tetroxide, dehydrated in alcohols and embedded in Araldite. The immunofluorescence procedure shows that non-immune serum does not label brain tissue (Fig. 1) which after this treatment exhibits only weak nonspecific fluorescence with some bright yellow autofluorescent granules in large neuronal perikarya. In contrast anti-S-100 induces labelling throughout the brain in cells which from their morphology and distribution are readily identified as glia. In the cerebral cortex (Fig. 2) astrocytes are selectively revealed, whereas in the cerebellar cortex (Fig. 3) it is the Bergman glial cells and their radial

Fig. 3 Cryostat section of cerebellar cortex treated with anti-S-100 serum for immunofluorescence. Perikarya of Bergmann glial cells (Bg) lying in the Purkinje cell layer are labelled as are the radial processes of these cells and their subpial endfeet at the surface of the cerebellar folia. Purkinje cell bodies (PC) are not labelled nor are the abundant granule cells in the layer subjacent to the Purkinje/Bergmann cell layer. Magnification, $\times 330$.



processes which are strikingly labelled. In all brain regions glial cells and their processes are also selectively stained by immunoperoxidase histochemistry with anti-S-100 (Fig. 4) by which means two classes of glial cells can be distinguished: those whose nuclei are stained by anti-S-100 and those whose nuclei are not so stained. With either immunohistochemical method no labelling of neuronal cell elements by anti-S-100 could be detected at the light microscopic level.

In the electron microscope, immunoperoxidase histochemistry is again capable of revealing glial cells and their processes, which are filled with the electron-dense peroxidase reaction product (Fig. 5). In neither cerebral nor cerebellar cortices did we find any neuronal cell bodies or processes, either axonal or dendritic, containing anti-S-100 induced reaction product.

We have paid particular attention to the postsynaptic junctional membrane where Haglid *et al.*¹⁰ claim to have localised S-100 antigen by immunoperoxidase histochemistry. The detection of electron-dense reaction product at this site is complicated by the presence of endogenous structures, the postsynaptic densities^{13,14}, which

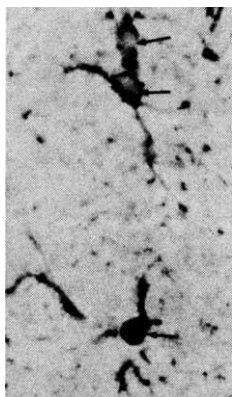


Fig. 4 Tissue slice of cerebral cortex treated with anti-S-100 in the immunoperoxidase procedure described in the text. Three glial cell bodies in this field show the immunoperoxidase stain (arrows) in one of which the nucleus is also stained. Various stained cellular processes are also visible. Magnification, $\times 400$.

as their name implies do themselves appear electron-dense after routine fixation for electron microscopy. To distinguish between two types of electron density at the same site is obviously difficult, and to maximise the difference between anti-S-100-induced peroxidase reaction product and endogenous synaptic density we have omitted the customary staining with uranyl or lead salts, both of which enhance the electron density of the postsynaptic density¹⁴. In these conditions it can be seen that although astrocytic processes in the neuropil, including those which invest the synapse, contain abundant anti-S-100 induced peroxidase reaction product, the synaptic junctional densities show only their endogenous density (Figs 5 and 6). Indeed the intimate relationship between glial processes and synaptic junctions is strikingly demarcated by this method, and astrocytic elements are seen to contain S-100 antigen where they surround both axon terminal and dendritic spine up to their abutment against the synaptic cleft (Fig. 6).

Our results thus contradict those of Haglid *et al.*¹⁰ in showing a complete absence of S-100 antigen from synaptic junctions in brain. Since glial S-100 in astrocytic processes immediately adjacent to the synaptic junction was visualised by our staining procedure, the apparent absence of S-100 from the junctions in our preparations cannot be attributed to failure of the staining reaction. On the other hand Haglid *et al.* have not presented evidence that their immunohistochemical preparations are capable of revealing

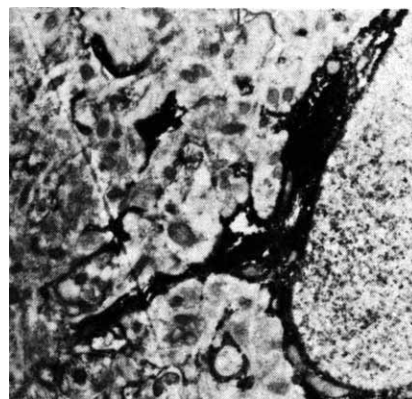


Fig. 5 Electron micrograph of cerebral cortex tissue stained with anti-S-100 by the immunoperoxidase procedure. A glial cell body and its processes are filled with immunoperoxidase reaction product. Magnification, $\times 5,000$.

the glial S-100 which constitutes the major portion of this protein in brain tissue. Since the glial S-100 has not been localised, their claimed anti-S-100 staining of the postsynaptic membrane must be subject to question.

Absence of S-100 from synaptic junctions is supported by available biochemical evidence. After reduction of disulphide bonds S-100 protein has a mobility in chromatographic procedures indicative of a molecular weight of 7,000 daltons (ref. 15). Electrophoretic analysis of isolated synaptic membranes and isolated synaptic junctional densities shows that protein components in this molecular weight range are absent^{16,17}.

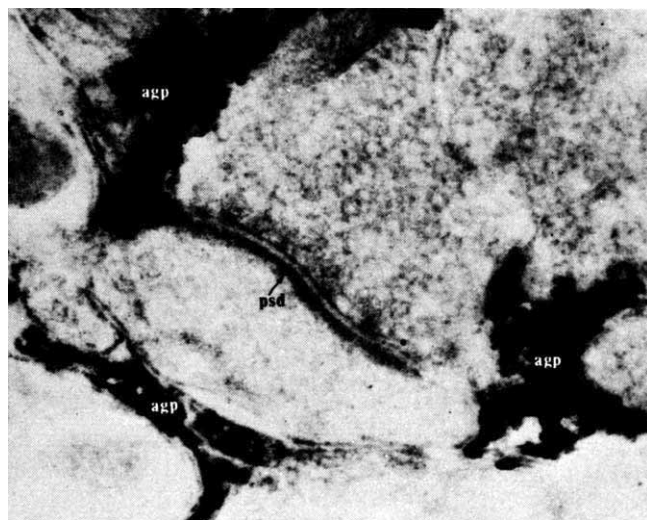


Fig. 6 A synapse in the cerebral cortex neuropil showing the staining obtained at all such sites with anti-S-100 by the immunoperoxidase procedure. Astroglial processes (agp) surrounding the axon terminal and dendrite are stained but the neuronal components of the junction are unstained. The junctional membranes show only their endogenous dense staining structures. No immunoperoxidase reaction product is associated with the postsynaptic density (psd). Magnification, $\times 62,500$.

Our results suggest that S-100 in brain is exclusively associated with glial cells. This implies that S-100 is not directly involved in chemical events occurring within neuronal cytoplasm or the synaptic membranes, although its presence in glial elements contiguous with the junction may be functionally significant. In particular the selective interaction which S-100 undergoes with Ca^{2+} and other

cations^{18,19} and the importance of Ca^{2+} in synaptic transmission may have some bearing on its presence at this site.

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MATUS and Mughal report a failure to localise S-100 protein in neuronal structures by the aid of immunofluorescence and immunoelectron microscopy. At present, the localisation in neuronal structures of the S-100 protein has been reported in 14 papers¹⁻⁷.

In describing their technique, Matus and Mughal state that they have used a "non-immune serum" diluted 1:50 for controls, and found only weak cytoplasmic autofluorescence of neurones with the immunofluorescence procedure. This is hardly a sufficient control since such procedures require careful testing of the experimental conditions as a basis for interpretation. For controls, they should have used antiserum against S-100, from which antibodies against S-100 had been removed by affinity chromatography or by absorption procedures¹⁻⁷. Seeing that immunohistochemical tests for the S-100 protein were used by Matus and Mughal, their negative results are open to doubt.

We would like to question another detail in their technique. Matus and Mughal have fixed the brain tissue with 4% formaldehyde and 0.5% glutaraldehyde (in the peroxidase procedure). This treatment will probably abolish the S-100 antigen-antibody reaction. We have found that 0.5% glutaraldehyde used for fixation of brain tissue for 1 h followed by thorough rinsing was detrimental to this antigen-antibody reaction⁷. Immunoelectrophoresis demonstrated furthermore that 0.5% glutaraldehyde added to a solution of S-100 protein completely inhibited S-100 antigen-antibody precipitation. Formaldehyde, 4%, decreased the reaction by 60% (ref. 7).

In their technical description Matus and Mughal do not report which measures and tests they have used to secure the specificity of the antiserum to S-100. We have stressed¹⁻⁷ the need to prepare a pure antigen (S-100) against which the antiserum can be tested. The purity of the S-100 was checked in sucrose density gradients and by electrophoretic methods. The antigen thus obtained was used to test the specificity of the antiserum to S-100. This specificity was tested in electrophoretic systems and by complement fixation¹⁻⁷.

Matus and Mughal point out that detection of differ-



Fig. 1 *a*, Synapse incubated in anti-S-100 antiserum conjugated with peroxidase. Heavy anti-S-100 peroxidase activity is seen in the postsynaptic membrane (arrow) ($\times 36,040$). *b*, Synapse incubated in anti-S-100 antiserum conjugated with peroxidase and repeatedly absorbed with pure S-100. No precipitates are seen in the postsynaptic membrane (arrow) ($\times 36,040$).

ences in electron density at the site of the postsynaptic membrane is complicated. We fully agree and consider that the only interpretable result could be judged, as the difference in electron density between sections treated with an antiserum containing antibodies against S-100 protein, and the same antiserum absorbed repeatedly with the antigen. This would ensure that the proper antibody has been studied in sufficient concentration.

In ref. 1 we studied exclusively neuronal S-100. The reason why we did not demonstrate much glial S-100 in subsequent work⁷ is that most soluble S-100 is lost to the medium from the cells during the mild fixation employed by us. Thus, the medium seems brownish after DAB incubation. We feel it necessary to stress that in our preparation only the small membrane-bound (less than 10%) portion of the total S-100 in the tissue is demonstrated.

Figure 1*a* shows a synapse from adult rat frontal cortex incubated in anti-S-100 antiserum conjugated with peroxidase. A heavy anti-S-100 peroxidase activity is seen in the postsynaptic membrane. Figure 1*b* shows a similar synapse incubated in anti-S-100 antiserum conjugated with peroxidase and repeatedly absorbed with pure S-100. No precipitates are seen in the postsynaptic membrane.

Our data do not, however, fully contradict Matus and

Mughal's results since some synapses do seem to lack S-100 activity⁷.

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Tyramine activates the EEG in epileptic patients

It has been suggested that abnormalities of monoamine metabolism may be important in migraine¹, and Sandler *et al.*² have demonstrated a reduction in monoamine oxidase activity in these patients. This observation might explain Hanington's finding³ that an oral tyramine load could induce headache in selected individuals. These subjects had noticed that certain foods known to contain tyramine, such as cheese, regularly induced their migraine headaches. Although unable to confirm that single doses of oral tyramine regularly precipitated headache³ we have shown that this amine accentuates pre-existing electroencephalogram (EEG) abnormalities in subjects with dietary-induced migraine alone, and in a group of patients who had both migraine and epilepsy, but not in a group with non-dietary migraine. These observations suggested that the effect of tyramine on the EEG should be studied in patients with epilepsy alone. Furthermore, a preliminary investigation had indicated that tyramine caused an increased number of spikes in the EEG recordings of monkeys with alumina-induced epileptic foci⁴.

We studied the effect of tyramine on the EEGs of a group of 15 patients with epilepsy using a double-blind placebo-controlled design. None of these subjects had migraine. Six women and nine men (mean age 36; range 16-63 yr) agreed to participate after the investigation had been explained to them. They suffered from generalised epilepsy of major or minor type or from temporal lobe epilepsy (Table 1). In most patients seizures were not well controlled. During the period of the investigation their usual anti-convulsant medication was continued. The trial required each patient to take a capsule on two separate occasions at least 1 week apart. One capsule contained tyramine hydrochloride (125 mg), and the other contained lactose placebo. EEGs were recorded approximately 5 h after ingestion of each capsule. All the recordings were made in a standard manner, at the same time of day, usually by the same technician, and using the same apparatus. During the 24 h after taking the test substances none of the 15 subjects complained of headache and none experienced seizures.

The 15 pairs of EEGs were masked, coded and randomised. Each pair was independently assessed by two raters using a previously determined procedure^{4,5}. In particular, disturbances of background activity and the occurrence of paroxysmal features, including spike activity, sharp waves, and spike and wave, were noted. All the EEGs were

Table 1 Activating effect of tyramine on EEG of epileptic patients

Generalised epilepsy	Activated 5	Not activated 0
Temporal lobe epilepsy	6	3
Total	11	3

$n = 14$

abnormal. The two raters agreed as to which was the more abnormal of a pair in 14 of the 15 instances. When the EEGs were unmasked it was found that in 11 of the 14 agreed assessments the recording made after the ingestion of tyramine was the more abnormal of the pair ($P < 0.01$). Accentuation of EEG abnormality after tyramine occurred both in patients with generalised epilepsy and in those with focal epilepsy (Table 1).

There are two separate problems in epilepsy to which these observations might be related. First, in epileptic patients there is a continuing subclinical disorder that is shown by the presence of abnormalities in inter-ictal EEG recordings and, second, there must be a triggering mechanism which induces clinical seizures at a particular moment. The underlying functional disturbances accounting for both these factors is uncertain although abnormalities of the cellular sodium pump⁶ and γ -aminobutyric acid metabolism⁷ have been suggested in experimental work. The present study showed that activation of pre-existing focal or generalised 'epileptic' EEG abnormalities was induced by oral tyramine. Although the type and quantities of these abnormalities are known to bear a direct relationship to seizure incidence^{8,9} none of our patients experienced seizures in the 24 h after ingestion of tyramine. We have not yet studied the effects of different doses of tyramine in these or other epileptic subjects.

Tyramine is a substrate for monoamine oxidase metabolism and it is possible that our findings could be related to a disturbance of that enzyme. This suggestion is supported by evidence that the seizure threshold in epileptic patients may be modified by changes in cerebral monoamines and that anti-convulsant drugs alter cerebrospinal fluid monoamine metabolite concentrations¹⁰. It is further supported by the fact that monoamine oxidase inhibitor drugs may precipitate seizures in some individuals¹¹ and by reported abnormalities in this enzyme in patients with another paroxysmal disorder of the brain, migraine². This is also a disorder in which EEG abnormality is present⁴. Disturbance of brain monoamine oxidase metabolism probably also has an important role in endogenous depression¹² and schizophrenia¹³, other disorders in which EEG abnormalities may occur.

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Disruption of brain stimulation-induced feeding by dopamine receptor blockade

It is well established that electrical stimulation of the lateral hypothalamus can be used to elicit a variety of biologically significant behaviour^{1,2}. For many years, this behaviour was usually attributed to the artificial activation of neural systems which control consummatory responses, but this interpretation has been called into question³. The criticism arises in part from behavioural experiments in which stimulation-induced feeding and drinking were shown to differ from their natural counterparts in a number of important respects^{4,5}, and also from neuropharmacological studies (ref. 6 and A.G.P. and H. C. Fibiger, unpublished) showing that food deprivation and brain stimulation-induced feeding or drinking were differentially affected by destruction of catecholamine (CA) systems in the brain. These latter results clearly implicated brain CAs in a stimulation-induced behaviour and as such emphasise the parallels between electrically-induced behaviour and stereotypies which accompany high doses of amphetamine⁷.

Amphetamine increases the release and prevents reuptake of both noradrenaline (NA) and dopamine (DA) and could therefore induce stereotypy by its action on any of the brain CA systems⁸⁻¹⁰. DA seems, however, to play a critical role in amphetamine stereotypy as this drug-induced behaviour is abolished by lesions of the dopaminergic nigrostriatal bundle^{11,12}. Furthermore, a similar class of stereotypical behaviour is elicited by the DA agonist apomorphine¹³. Therefore, if brain stimulation and drug-induced behaviour are subserved by a common neurochemical substrate, the former behaviour should be disrupted by treatment with a DA antagonist. As a test of this hypothesis, we observed the effects of the neuroleptic drug haloperidol on feeding elicited in satiated rats by electrical stimulation of the lateral hypothalamus. In small doses, haloperidol has been reported to block central DA receptors¹⁴.

Twenty-five male Wistar rats had bipolar electrodes im-

planted into the lateral hypothalamus under stereotaxic control. Implantation procedure and behavioural testing were carried out as described previously⁸. Briefly, the behavioural testing consisted of first selecting a current intensity which induced forward locomotion and searching, and observation of each animal for five daily test sessions, consisting of twenty 30-s periods of brain stimulation, separated by 60-s interstimulation intervals. The six animals which showed brain stimulation-induced feeding behaviour during seven or more stimulation periods on a given day, were retained for further testing.

During the next phase of the experiment, these six animals were tested for 5 d per week. Three days of testing preceded the drug treatment, and one day of testing followed it. On day 3 of each week, the animals were injected with saline (0.9%), on day 4 they were injected intraperitoneally with either 0.05, 0.10 or 0.15 mg kg⁻¹ haloperidol (Haldol, McNeil Laboratories) 45 min before beginning the test series. Measurements of the total time engaged in stimulation-induced behaviour, and the amount of wet mash ingested were taken, together with the number of trials on which the behaviour was displayed. During the next week, the effect of haloperidol (0.15 mg kg⁻¹) on deprivation-induced food intake, was observed. The amount of food consumed in 1 h following 23 h food deprivation was measured for 4 d and on the day 5, the food intake after pretreatment with haloperidol was monitored. At the end of week 4 of testing, the animals were asphyxiated in CO₂, their brains removed, sectioned on ice, and electrode placements confirmed microscopically. The six electrodes were all located in the lateral hypothalamus, with five situated dorsolateral to the fornix, on the edge of the cerebral peduncle.

The data from each of the three measures are shown in Table 1. Treatment with the DA receptor blocker haloperidol produced a disruption of stimulation-induced feeding, which followed a clear dose-response relationship. None of the three measures of stimulation-induced feeding was affected by the lowest dose of haloperidol (0.05 mg kg⁻¹) and only a slight decrease was observed after 0.10 mg kg⁻¹. A significant decrement accompanied the highest dose (0.15 mg kg⁻¹), however, as reflected in a 74% reduction in incidence ($P < 0.01$), 76% reduction in time ($P < 0.01$) and 70% decrease in amount of food consumed ($P < 0.01$). In contrast, this same dose of haloperidol had no significant effect on the feeding behaviour of deprived animals, confirming a recent report¹⁵. Food intake during the no-drug condition was 35 g wet mash, whereas the

Table 1 Effect of haloperidol on stimulation-induced feeding as measured by incidence, time and amount of food consumed

Measures	Saline	Haloperidol (0.05 mg kg ⁻¹)	Treatment		Saline	Haloperidol (0.15 mg kg ⁻¹)
			Saline	Haloperidol (0.10 mg kg ⁻¹)		
Incidence of stimulation-induced feeding (maximum 20)	13 ± 4.4	12.8 ± 6.9	13.6 ± 4.1	10.2 ± 6.8	11.3 ± 3.8	3.0 ± 1.9*
Total time engaged in stimulation-induced feeding (maximum 600 s)	81.7 ± 36.2	86.2 ± 65.2	90 ± 40.8	58.6 ± 47.8	57.5 ± 20.8	13.8 ± 10.7*
Amount of food (g) consumed during 20 stimulation periods	25.5 ± 9.2	26.5 ± 17.5	27.2 ± 11.6	20.3 ± 11.9	21.5 ± 4.8	6.5 ± 4.3*

Data expressed as mean ± s.d.

$P < 0.01$ when compared with saline by analyses of variance and *post hoc* Student's *t* test.

same animals consumed 30 g in 1 h following 0.15 mg ml⁻¹ haloperidol ($P > 0.05$).

Previous experiments have implicated brain CAs in stimulation-induced behaviour and the present results suggest an important function for DA. Dopaminergic mechanisms are thought to be involved in the homeostatic regulation of food and water intake¹⁶⁻¹⁸, although the precise nature of this involvement is unknown. Theoretically, feeding and drinking are controlled by a number of different subsystems and in this context, it is of interest to note that whereas haloperidol has no effect on food deprivation-induced feeding, eating in response to insulin is attenuated by this drug⁶. It is, therefore, conceivable that brain stimulation-induced feeding may be subserved by activating one or more specific subsystems normally involved in the regulation of food intake. If brain stimulation-induced feeding is in some way dependent on neural systems controlling *ad libitum* food intake, however, the relationship seems to be a limited one in view of the differential effects of both haloperidol and 6-hydroxydopamine (ref. 6 and A.G.P. and H. C. Fibiger, unpublished) on these different types of feeding behaviour.

As an alternative explanation, it is compelling to consider brain stimulation-induced behaviour as representative of a general class of stereotypical behaviours which include drug-induced stereotypes^{7,13} and consumatory responses evoked by mildly arousing stimuli such as tail-pinch in rats¹⁹. Compulsive oral movements including licking and gnawing, are characteristic of each class of induced behaviour and these responses are attenuated by disruption of dopaminergic activity regardless of the type of stimulation used to evoke them. Although the present data do not permit specification of a particular dopaminergic substrate for brain stimulation-induced behaviour, both tail-pinch and amphetamine-induced stereotypes seem critically dependent on the nigro-neostriatal bundle^{12,13,19}. This system may also be important for electrically-induced behaviour, as preliminary results in our laboratory indicate that such behaviour is disrupted by neurotoxic lesions of the nigro-neostriatal bundle. It has been suggested that the nigro-neostriatal bundle serves an important gating function in the control of neostriatal motor output²⁰, and therefore disruption of this system could result in the release of prepotent motor responses normally inhibited by the neostriatal bundle. Valenstein²¹ has proposed that behaviour induced by brain stimulation, tail-pinch and amphetamines could represent coping responses initiated by a state of high arousal and hyper-responsiveness to stimuli, resulting from facilitation of DA pathways. Although these explanations undoubtedly represent an oversimplified statement of the role of dopamine in stereotype behaviour, this perspective could provide important insights into stereotype behaviour in general, and brain stimulation-induced behaviour in particular.

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Organisation of receptors for neurotransmitters on *Aplysia* neurones

THE application of acetylcholine (ACh) to neurones of *Aplysia* has shown the existence of three different responses, mediated by conductance increases to Na⁺, Cl⁻ and K⁺ respectively¹. These neurones also have receptors for various other putative neurotransmitters, including serotonin², dopamine³, octopamine^{4,5}, phenylethanolamine⁶, γ -aminobutyric acid^{6,7}, glutamic acid^{6,7}, aspartic acid⁷ and histamine^{5,8}. With one possible exception there are at least three types of response to each of these substances resulting from conductance increases to Na⁺, Cl⁻ and K⁺, respectively (refs 5-7 and our unpublished observations). These facts suggest that specific receptors (neurotransmitter-binding sites) and ionophores for Na⁺, Cl⁻ and K⁺ might be building blocks which can be assembled in any combination. If so there should be common properties of the receptors for one transmitter mediating the three different ionic responses, as well as similarities in the responses to different transmitters when the conductance change is to the same ion. In this report we compare the three dopamine and ACh responses as a test of this hypothesis.

Neurones were penetrated with double-barrelled glass microelectrodes as previously described^{4,8,9}. One barrel was used to record membrane potential and the other to pass d.c. or current pulses for measurement of membrane resistance. Drugs were iontophoresed using a five-barrelled extracellular micropipette. The iontophoretic control unit was designed to pass the same total charge through each of the five barrels, and would automatically vary pulse duration to achieve constant total charge (J. A. Willis, in preparation). For substances with similar transport numbers this method allows a comparison of the sensitivity of a receptor to equal amounts of different substances applied to a localised site.

Figure 1 shows examples of the three different conductance increase responses to dopamine. Each of these three neurones was studied with iontophoretic electrodes containing noradrenaline, octopamine and phenylethanolamine in addition to dopamine. Figure 1a shows a neurone responding to dopamine with a large depolarisation which causes a brisk discharge. This neurone also responds to noradrenaline but not as well as to dopamine. In the absence of Na⁺ (replacement with the impermeant Tris⁺) there is no response, indicating that the depolarisation is a result of a conductance increase to Na⁺. In Fig. 1b another neurone is hyperpolarised best by dopamine and less well by noradrenaline. This response is due to a Cl⁻ conductance increase, since removal of extracellular Cl⁻ (replacement by impermeant acetate) causes a reversal of the polarity of the response due to the reversal of the Cl⁻ concentration gradient. In another neurone (Fig. 1c) a similar relative sensitivity to dopamine and noradrenaline results for a response unaffected by removal of extracellular Cl⁻ and identified by Ascher³ as being due to a K⁺ conductance increase. In all three neurones there was no response to octopamine or phenylethanolamine although other neurones have receptors for these substances^{4,5}. Adrenaline, applied to the bath at a concentration of 10⁻⁴ M, caused a con-

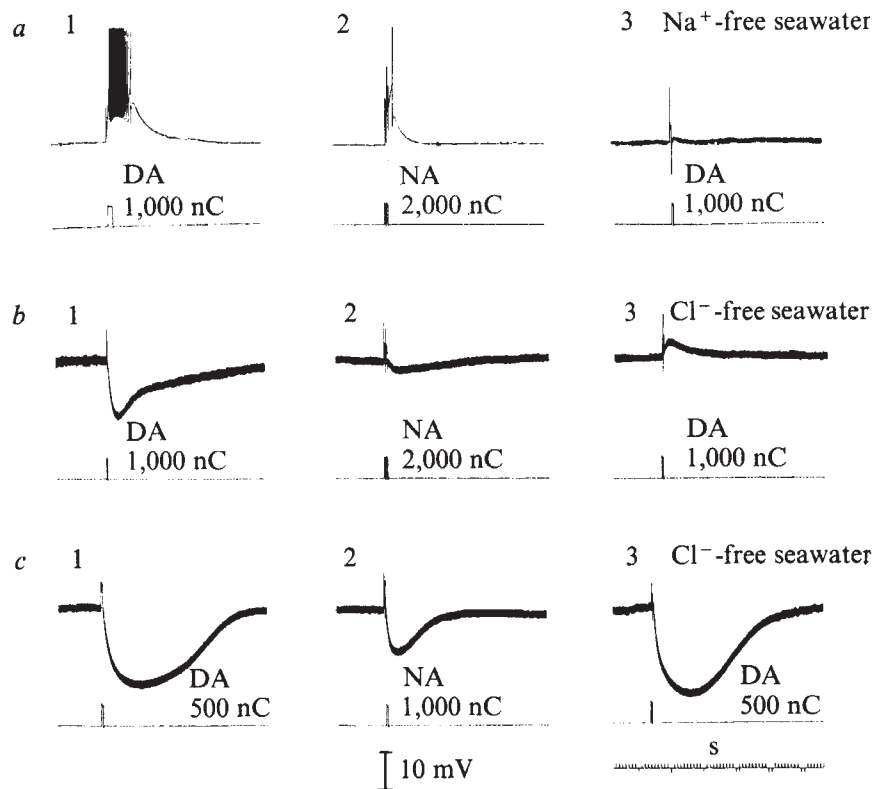


Fig. 1 Effects of iontophoretic application of dopamine and noradrenaline on to three different unidentified neurones from the abdominal (*a* and *b*) and cerebral (*c*) ganglia. The upper recording in each trace is from the intracellular micropipette, whereas the lower trace shows the duration of the iontophoretic pulse. The total charge passed by iontophoresis is indicated near the pulse. In *a*3 the ganglion was perfused with seawater where Na^+ was replaced by Tris^+ , whereas in *b*3 and *c*3, all Cl^- was replaced by acetate. The time calibration applies as shown only to *a* and *b*. In *c* the time base is slower by a factor of 2. DA, dopamine; NA, noradrenaline.

ductance change similar to but smaller than that caused by dopamine and noradrenaline. In every case, membrane resistance fell during the response. Figure 2 shows that when the dopamine receptor is desensitised there is no longer a response to noradrenaline. We conclude that both substances are acting on a common receptor, and that the structure-activity relation of that receptor is identical for all three ionic responses.

Table 1 gives the average time-to-peak of the Na^+ , Cl^-

and K^+ responses to dopamine and ACh. Neurones were unselected except for the requirement of clear identification of the ionic conductance involved by changing the seawater composition as illustrated in Fig. 1 and/or by obtaining an inversion potential at close to levels previously reported¹ (-60 mV for E_{Cl^-} , -80 mV for E_{K^+}). For both dopamine and ACh the Na^+ and Cl^- responses are very much more rapid than the K^+ responses. The values for the same ionic conductance to the different transmitters are very similar.

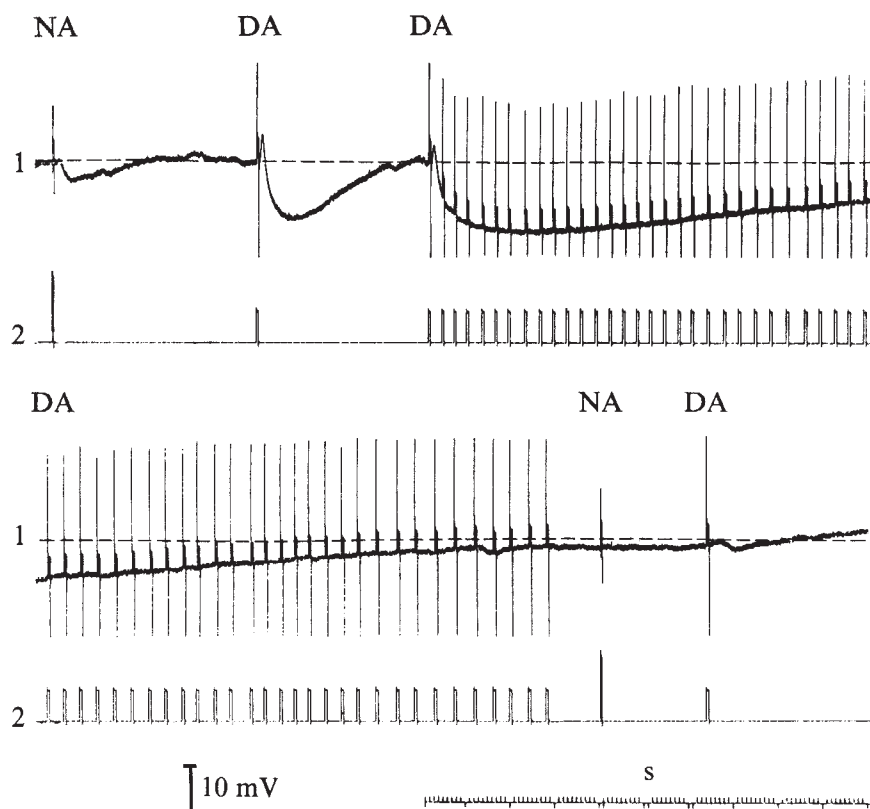


Fig. 2 Effects of dopamine receptor desensitisation on the response of a pleural neurone to noradrenaline. Intracellular recordings are shown in trace 1, whereas trace 2 indicates the iontophoretic pulses. The upper and lower traces are continuous. In trace 2 the large pulse indicates noradrenaline application while the smaller one indicates dopamine. The total charge was 1,000 nC for both dopamine and noradrenaline. Multiple pulses of dopamine resulted in a hyperpolarisation which decayed towards control potential with time. In these conditions membrane resistance returned to control values. When the receptor was desensitised to dopamine there was no longer a response to noradrenaline, indicating that both act on the same receptor.

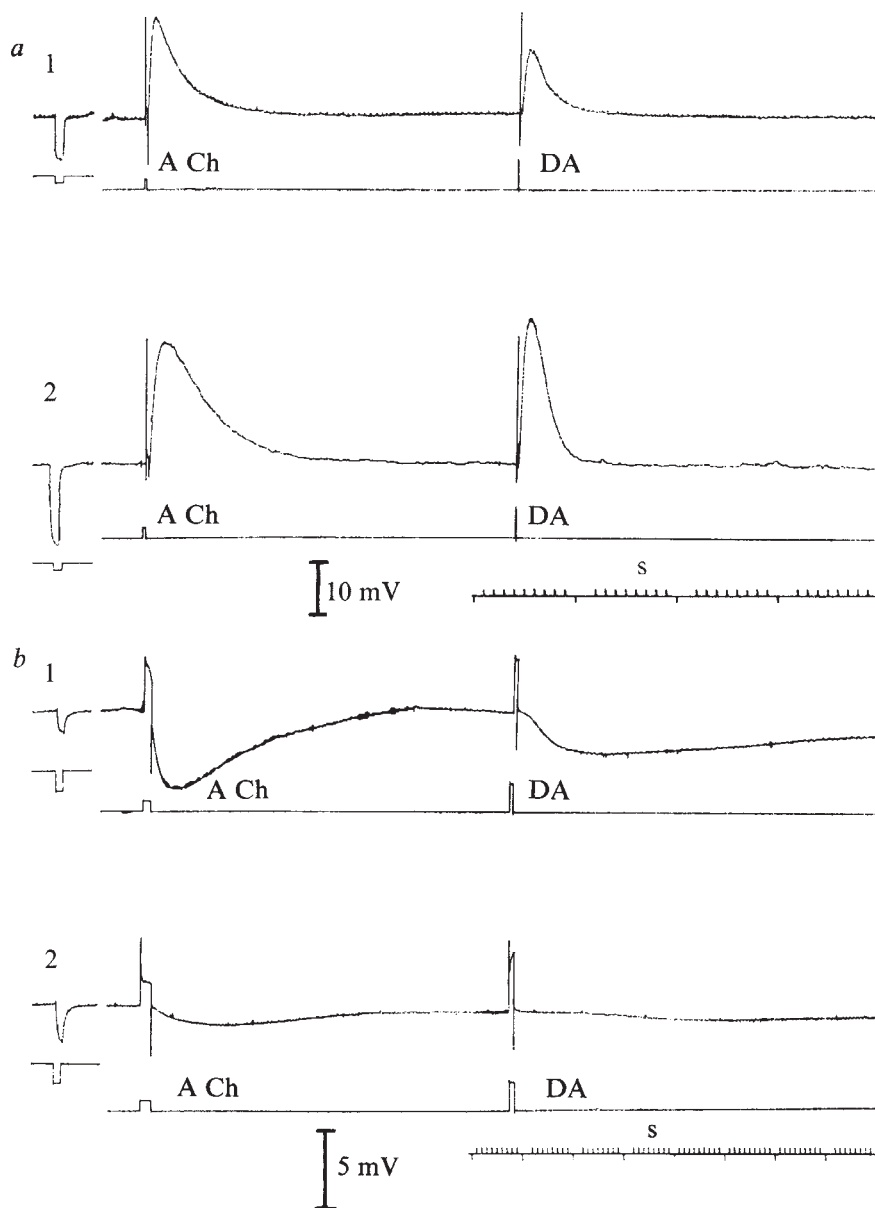


Fig. 3 Effects of temperature on Na^+ and K^+ responses to ACh and dopamine. In each recording the upper trace is the intracellular potential and the lower is the indication of the iontophoretic pulse. The small inserts at the left of each row show a measure of membrane resistance (upper) to a current pulse of 1.2×10^{-8} A in (a) and 4.0×10^{-8} A in (b). Records a1 and b1 were made at 24°C , and records a2 and b2 at 8°C . Temperature changes and methods of recording were as previously described¹¹. The neurone in (a) was from the anterior pleural group described by Kehoe¹, whereas (b) was from cell R_{14} in the terminology of Frazier *et al.*¹². For both neurones penetrations were made with two independent intracellular electrodes (one for recording, the other to pass current). Iontophoretic current was 100 nC in (a) and 1,000 nC in (b) for both dopamine and ACh. For R_{14} penetration and iontophoresis were made through the capsule.

In all cases the dopamine response is slightly slower than that to ACh, but this, in part at least, reflects the fact that all dopamine receptors are located in the neuropil (in agreement with Ascher³), whereas ACh receptors are both on soma and neuropil.

The ionic selectivity of the K^+ ionophore has been studied for both ACh and dopamine receptors. Kehoe¹ has shown that ACh K^+ ionophore is impermeable to Cs^+ but one half as permeable to Rb^+ as K^+ . Ascher³ has found a similar pattern for the dopamine K^+ ionophore. In addition, Kehoe¹ has demonstrated that intracellular TEA injections block the ACh K^+ responses (presumably by occluding the K^+ channel¹⁰ rather than by blocking the ACh receptor complex, as it does when applied extracellularly). Ascher³ demonstrates a similar sensitivity of the dopamine K^+ ionophore to intracellular but not extracellular TEA.

Figure 3 illustrates the temperature dependences of Na^+ and K^+ responses. The neurone in Fig. 3a gave a Na^+ conductance to both ACh and dopamine. On cooling from 23°C (1) to 8°C (2) the membrane resistance increased considerably and the responses increased proportionately. In Fig. 3b, K^+ responses to both ACh and dopamine were reduced considerably by cooling in spite of an increased resistance. These results are in agreement with others^{1,3}. Although we have found exceptions to the generalisation

that all Na^+ responses are relatively temperature insensitive and all K^+ responses are blocked by cooling, the temperature dependence of the same ionic responses elicited by two different transmitters on the same cell has always been the same.

Shain *et al.*⁹ suggested that *Aplysia* contains a single ACh receptor on the basis of observations that α -bungarotoxin blocks all three ACh responses and binds to a membrane homogenate with a single association and dissociation constant. Our observation that the various phenylethylamines have similar relative effectiveness in activating the three dopamine responses is consistent with the view that the dopamine binding site is identical irrespective of the conductance change elicited. The similarities in time course,

Table 1 Times-to-peak of Na^+ , Cl^- and K^+ responses to ACh and dopamine

	Na^+	Cl^-	K^+
ACh	1.8 ± 0.2 (36)	2.4 ± 0.2 (43)	12.2 ± 1.7 (6)
Dopamine	2.3 ± 0.7 (14)	2.5 ± 0.5 (7)	15.1 ± 1.5 (21)

Results are expressed in $s \pm \text{s.e.}$ The values in parentheses indicate the number of neurones of each group studied.

temperature sensitivity and ionic selectivity of responses resulting from similar ionic conductance but activation of receptors to different transmitters, suggest that these properties are characteristic of common ionophores.

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Lithium accumulation by snail neurones measured by a new Li^+ -sensitive microelectrode

THE importance of lithium salts in the treatment of mania¹ has led to considerable research into their physiological and biochemical effects, especially on the nervous system. But, largely because of analytical difficulties, little is known about Li^+ accumulation or transport by nerve cells: the available evidence suggests only that there is some Li^+ uptake by neurones², but little or no active transport of Li^+ by the sodium pump³. The ideal method for measuring intracellular ions is the ion-sensitive microelectrode, but no electrode suitable for Li^+ was available until the recent development of a synthetic Li^+ -selective ligand⁴; thus we are now able to make Li^+ -sensitive microelectrodes. Here we describe their construction and use in the direct measurement of intracellular Li^+ in snail neurones. We have found that the neurones can maintain lower levels of Li^+ inside than out, showing that Li^+ must be actively transported out across the cell membrane.

In early experiments, the Li^+ -selective ligand was used to make macroelectrodes by incorporation in a polyvinylchloride (PVC) membrane using Tris-(2-ethylhexyl)-phosphate (TEHP) as plasticiser. The response characteristics of such a membrane are shown in Fig. 1 (refs 4 and 5). According to Fig. 1, Na^+ is discriminated relative to Li^+ by a factor ~ 20 . So that the same Li^+ carrier could be used in microelectrodes with reasonably low ($\sim 10^{10} \Omega$) resistances, sodium tetraphenylborate (NaTPB) was added to the membrane phase. The Li^+ -ligand solution had the following composition (by weight) 9.7% Li^+ carrier, 85.5% TEHP and 4.8% NaTPB .

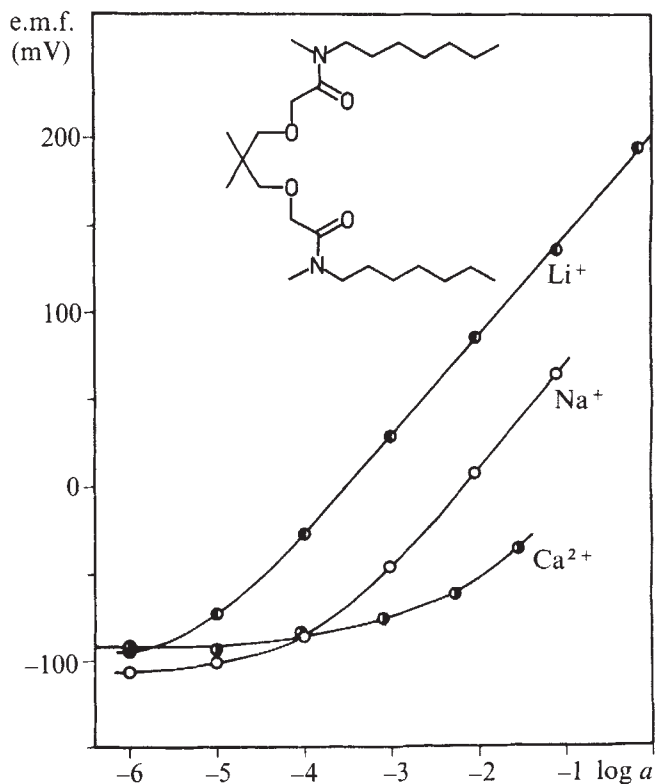
The Li^+ -sensitive microelectrodes were made from borosilicate glass micropipettes by a modification of one of the methods of Walker⁶ (P. Ascher and T. O. Neild, personal communication). The micropipettes were siliconised by dipping for ~ 10 s in a 2.5% v/v solution of tri-*n*-butyl chlorosilane in chloronaphthalene and then heated for at least 20 min at $\sim 100^\circ\text{C}$. The last few millimetres of the micropipette were filled with the Li^+ -ligand solution, air bubbles being removed with a cat's whisker (from a real cat) and the rest of the electrode being filled with 2 M LiCl . A chloride-coated silver wire, dipped in the LiCl , completed the microelectrode.

The physiological experiments were done on the largest neurone at the rear of the right pallial ganglion of the snail *Helix aspersa*⁷. The exposed suboesophageal ganglia were

superfused with continuously flowing snail Ringer (KCl , 4 mM; NaCl , 80 mM; CaCl_2 , 7 mM; MgCl_2 , 5 mM; NaHCO_3 , 22 mM; $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ pH 7.5, 0.1 mM; equilibrated with 2.5% CO_2 and 97.5% O_2). The neurone was penetrated with up to four separate microelectrodes: one to measure the membrane potential, one sensitive to Li^+ , and two for interbarrel iontophoretic injections of LiCl . After an experiment, the Li^+ -sensitive electrode was calibrated in the experimental bath by the perfusion of solutions with approximately the intracellular levels of Na^+ and K^+ , but different levels of Li . This method avoided the need for complex calculations using selectivity coefficients determined for each electrode. (It should be mentioned that ion-sensitive electrodes measure activity rather than concentration. But since the present experiments were done with an essentially constant ionic strength, the more convenient ion concentrations will be used.)

To check that this calibration method works reasonably well, a Li^+ -sensitive microelectrode was calibrated intracellularly as well as extracellularly. A series of LiCl injections were made while the internal Li^+ concentration ($[\text{Li}^+]_i$) was measured with a Li^+ -sensitive electrode, as shown in Fig. 2. At the beginning of the experiment there was no Li^+ inside the cell. When the fourth microelectrode (filled with LiCl) was inserted into the cell there was at first some leakage, but this was stopped by a 5-nA backing current. Five separate 35-nA injections of LiCl were then made, all except the fourth with current duration of 30 s. The neurone diameter was about 200 μm , so that, assuming a spherical cell and a transport number of 1.0, the 30-s injections should each raise $[\text{Li}^+]_i$ by 2.6 mM. The observed increases in $[\text{Li}^+]_i$ were measured using the extracellular calibration curve. The ratios of the increases in $[\text{Li}^+]_i$ calculated from the charge to those measured were 0.96, 1.15, 1.19, 1.21 and 1.15. This suggests that while the cell volume was presumably less than estimated, the

Fig. 1 E.m.f. response of a lithium-selective PVC membrane electrode to aqueous solutions of the chlorides of Li^+ , Na^+ and Ca^{2+} . The e.m.f. values were corrected for changes in the liquid junction potential of the reference electrode (see ref. 4). The structure of the electrically neutral Li^+ selective carrier is shown at the top.



Li^+ -sensitive microelectrode gave very similar responses inside and outside the cell. We found that the Li^+ -sensitive microelectrodes were usable for at least 2 d after filling, and were very stable in the calibration solutions or intracellularly. But in the snail Ringer the potential of the electrode tended to vary, in particular the potential after a cell penetration tended to differ from that before.

A simple experiment to measure Li^+ uptake and loss is shown in Fig. 3. The neurone was initially in normal Ringer. When this was changed to one with half the Na^+ replaced by Li^+ , the Li^+ record immediately began to rise, indicating a relatively slow Li^+ influx. After about 17 min in Li^+ Ringer, the cell stopped firing. When the cell was again exposed to normal Ringer, Li^+ ions began to leave the cell. In similar but longer experiments essentially all the accumulated Li^+ left the cell within 1 h. The $[\text{Li}^+]_i$ at which the spontaneous action potentials ceased was always $\sim 5 \text{ mM}$.

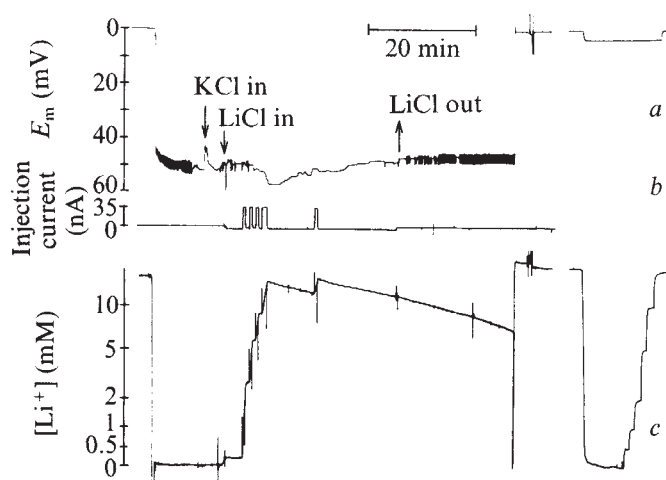


Fig. 2 Recordings comparing the Li^+ -sensitive microelectrode response to increasing $[\text{Li}^+]_i$ inside a neurone with that to calibration solutions. *a*, Potential between the membrane potential (E_m) microelectrode and a reference electrode in the bath; *b*, ionophoretic injection current; *c*, potential between the E_m and the Li^+ -sensitive microelectrodes. The Li^+ -sensitive microelectrode was inserted first, then the membrane potential microelectrode, and finally two current-passing microelectrodes: one filled with KCl and the other with LiCl. The long time constant of the pen recorder has reduced the size of the action potentials to only a few millivolts. At the end of the experiment the electrodes were withdrawn from the cell and the Li^+ microelectrode calibrated by superfusing the preparation with six different calibration solutions. These all had the same levels of NaCl (4 mM), KCl (90 mM) and Tris maleate pH 7.5 (10 mM), but different levels of LiCl (0, 0.5, 1, 2, 5 and 10 mM).

Perhaps the most interesting question that can be easily answered with a Li^+ -sensitive microelectrode is how much Li^+ cells accumulate when exposed to low, 'therapeutic' levels of external Li^+ . With a purely passive distribution of Li^+ across the cell membrane, 1 mM Li^+ outside would lead to a $[\text{Li}^+]_i$ of 5–10 mM. A number of experiments were therefore done in which cells were loaded with Li^+ by a brief exposure to high external levels, and then allowed to equilibrate in Ringer with only 1 or 2 mM LiCl. The graph of $[\text{Li}^+]_i$ against time from such an experiment is shown in Fig. 4. After a short exposure to 45 mM Li^+ Ringer, the cell was maintained in 1 mM Li^+ Ringer until the end of the experiment. Since the membrane potential was $\sim 45 \text{ mV}$, a passive distribution of Li^+ across the cell membrane would lead to intracellular accumulation of Li^+ to a level of more than 5 mM; yet the result shows that $[\text{Li}^+]_i$ fell to below 0.3 mM. Thus Li^+ was being extruded against both an electrical and a concentration gradient; in some way Li^+ was being actively transported out of the cell. This was also seen when cells were exposed to 2 mM or 10 mM external Li^+ ; with 2 mM Li^+ outside the

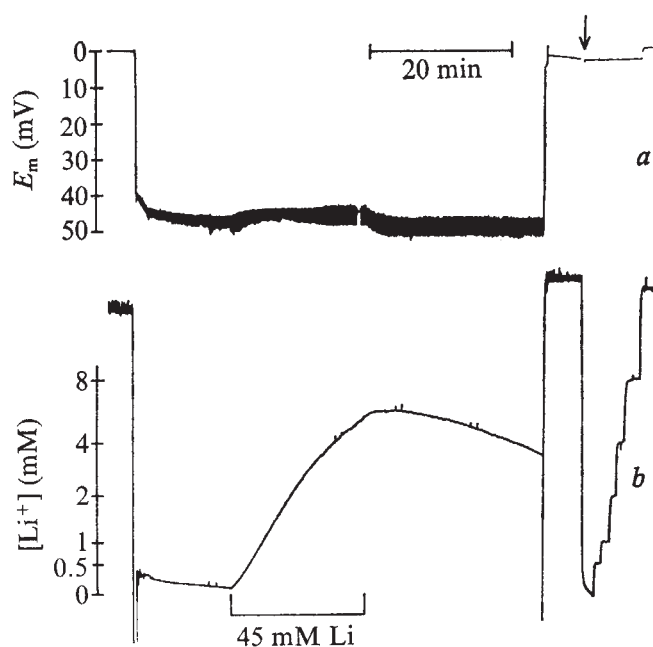
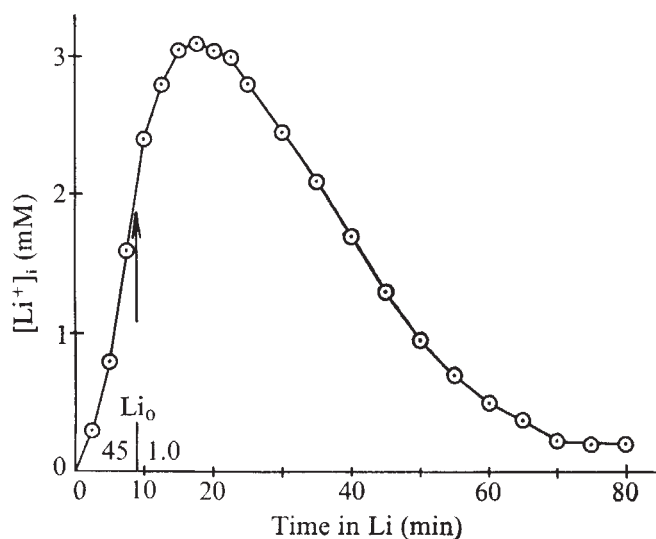


Fig. 3 Li^+ uptake by a neurone exposed to 45 mM external Li^+ . *a*, Membrane potential; *b*, potential of the Li^+ -sensitive microelectrode. The cell was penetrated by two microelectrodes only. Where indicated, the normal Ringer was changed to one in which half the Na^+ was replaced by Li^+ . At the end of the experiment both electrodes were withdrawn from the cell and the Li^+ electrode was calibrated. The six calibration solutions all had the same concentration of NaCl (2 mM), KCl (90 mM) and Tris maleate (10 mM), but different concentrations of LiCl (0, 0.5, 1, 2, 4 and 8 mM). At the point indicated by the arrow the E_m microelectrode blocked and was then broken to a low resistance.

steady state $[\text{Li}^+]_i$ was $< 1 \text{ mM}$, and with 10 mM Li^+ outside $[\text{Li}^+]_i$ was $\sim 5 \text{ mM}$. These results show clearly for the first time that Li^+ can be actively transported out of nerve cells. Similarly, frog muscle has recently been found⁸ by chemical analysis to accumulate very little Li^+ when exposed to low external levels. The mechanism by which Li^+ is actively extruded is unknown: perhaps the selectivity of the Na^+ pump in favour of Na^+ against Li^+ is not as high as has been thought^{3,9}.

The low levels of $[\text{Li}^+]_i$ accumulated with extracellular levels in the therapeutic range cast some doubt on a recent suggestion,

Fig. 4 Graph of the internal Li^+ concentration against time from an experiment in which a neurone was first exposed to a Ringer containing 45 mM Li^+ , and then to one containing only 1 mM Li^+ .



by Partridge and Thomas⁷ that an increase in potassium conductance caused by intracellular Li^+ might be related to its psychiatric effects. It is perhaps possible that the 'Li⁺ pump' varies in different neurones so that some might accumulate more Li^+ than others.

These results show that the new Li^+ -sensitive microelectrode works well intracellularly, and suggest that it will be an important tool in future Li^+ research. It is clear that Li^+ ions are actively transported out of snail neurones, so that at low external levels there is even less Li^+ intracellularly than extracellularly. This makes it harder to see how Li^+ could act intracellularly to change neuronal properties in Li^+ therapy.

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Experimental evidence that polyoma-specific tumour antigen is a virus-coded protein

POLYOMA virus (PV) induces intranuclear tumour (T) antigen formation in permissive and semipermissive cells. In virus-infected cells, T antigen appears before the onset of viral DNA synthesis and is continuously demonstrable in PV-transformed cells¹. The biological function of the T antigen is still uncertain, but the temporal relationship between early virus-specific messenger RNA (mRNA) synthesis, T antigen formation and stimulation of host cell DNA synthesis led to the hypothesis that T antigen acts as an activator for DNA synthesis^{2,3}. Furthermore there exists only indirect evidence that T antigen is a virus-coded protein¹. We have tested this by a more direct experimental approach. For this reason PV DNA was transcribed *in vitro*, and the complementary RNA (cRNA) obtained was further purified and injected into mouse tissue culture cells by our microinjection technique^{4,5}. PV-specific T and V antigen formation was investigated.

PV DNA I was used as template for the *Escherichia coli* DNA-dependent RNA polymerase. After transcription viral DNA was thoroughly removed by exhaustive DNase treatment (M.G. and A.G., unpublished). To prevent any contamination with viral DNA, the cRNA preparation was then centrifuged to equilibrium in Cs_2SO_4 at a density excluding the entrance of PV DNA into the gradient (M.G. and A.G., unpublished and ref. 6) (Fig. 1).

The size of the cRNA was estimated by formaldehyde-sucrose gradient centrifugation. Figure 2 shows that more than 50% of the PV cRNA has a sedimentation coefficient of 26S or more. The complete transcription product of one

PV DNA strand would have a molecular weight of 1.6×10^6 , sedimenting as 26S RNA in these centrifugation conditions⁸. *E. coli* RNA polymerase transcribes both the early and the late strand of the non-defective wild-type PV DNA I⁹. Depending on the preparation 60-80% of the cRNA

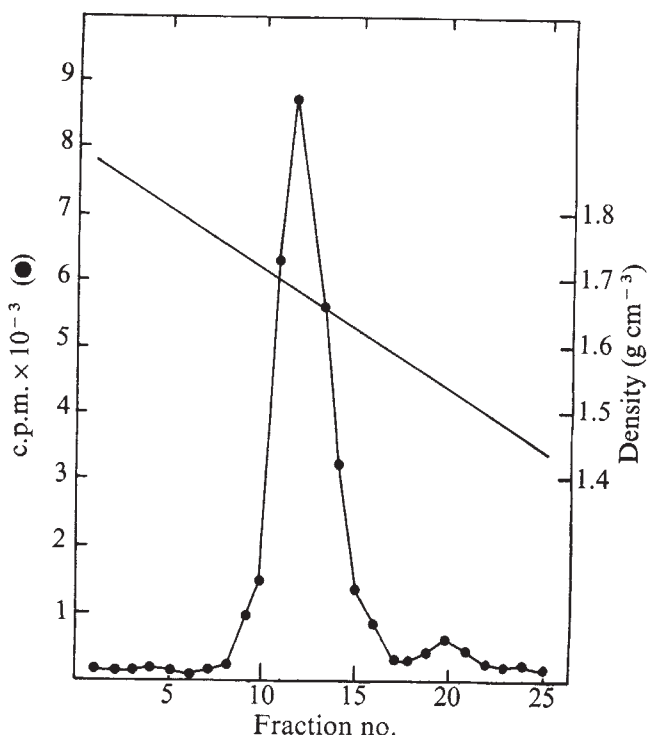


Fig. 1 Cs_2SO_4 equilibrium density gradient centrifugation of PV cRNA. Cultures of confluent primary mouse kidney cells were infected with plaque-purified (three times) wild-type virus (PV78) at a multiplicity of 0.1 PFU per cell. After an adsorption period of 2 h cells were washed and covered with serum-free Eagle's medium. PV DNA was extracted by the extraction method of Hirt⁷. Purification of PV DNA involved RNase treatment (Worthington, 50 $\mu\text{g ml}^{-1}$, 30 min, 37 °C), neutral sucrose gradient centrifugation (5-20% w/v, 1 M NaCl, 0.001 M EDTA, 0.001 M Tris-HCl, pH 7.4) and CsCl -ethidium bromide equilibrium centrifugation (1.56 g ml^{-1} CsCl , 100 $\mu\text{g ml}^{-1}$ ethidium bromide, 0.02 M Tris-HCl, pH 8.0)⁸. PV DNA I was transcribed with *E. coli* DNA-dependent RNA polymerase (Miles Laboratories, *E. coli* K12, grade II). The transcription reaction mixture (400 μl) contained 10 μg PV DNA I, 10 μg RNA polymerase, 0.15 M KCl, 0.04 M Tris-HCl, pH 8.0, 5% glycerol, 0.01 M MgCl_2 , 1.5 mM 2-mercaptoethanol, 0.4 mM each of ATP, GTP, CTP, UTP and 0.01 mM 8-³H-GTP (Amersham Buchler, 15 Ci mmol^{-1}). Nucleoside triphosphates were added after preincubation of PV DNA and RNA polymerase at room temperature. After 60 min incubation at 37 °C, 20 μg electrophoretically purified DNase (Worthington) was added to the reaction mixture and further incubated for 30 min at 37 °C. Then $0.1 \times \text{SSC}$ and 5% SDS were added to a final concentration of $0.01 \times \text{SSC}$ and 1% SDS. The resulting mixture was extracted twice with one volume phenolhydroxyquinoline (80% phenol in H_2O v/v and 1% hydroxyquinoline w/v). The cRNA preparation was then subjected to Cs_2SO_4 equilibrium density gradient centrifugation. The initial density of Cs_2SO_4 in a solution of EDTA-Tris (0.001 M EDTA, 0.01 M Tris-HCl, pH 7.2) was 1.62 g cm^{-3} . The gradient was centrifuged in a Spinco SW65 rotor at 45,000 r.p.m. for 48 h at 5 °C. The gradient was fractionated from the bottom of the tube and fractions 10-14 collected. Cs_2SO_4 was removed by passing the pooled fractions through a Sephadex G-25 column and by exhaustive dialysis⁸. The average yield of cRNA was 40-50 μg with a specific activity of $1-2 \times 10^4$ c.p.m. per μg RNA. Control experiments were made with 10 μg $\text{CH}_3\text{-}^3\text{H}$ -thymidine-labelled PV DNA (1.9×10^5 c.p.m.; 1.9×10^4 c.p.m. per μg DNA) which were transcribed as stated above (without 8-³H-GTP): a, after DNase treatment the cRNA preparation contained 4×10^3 c.p.m. in acid-precipitable material; b, after Cs_2SO_4 equilibrium density gradient centrifugation pooled fractions 10-14 contained less than 100 c.p.m. in acid-precipitable material equivalent to 0.05% initial DNA.

remained RNase resistant in self-annealing experiments (data not shown).

The biological activity of the cRNA was assayed in secondary mouse tissue culture cells after cRNA microinjection using our microinjection (glass capillary) technique^{4,5}. The volume transferred into the cytoplasm of the recipient cells was about 10^{-11} – 2×10^{-11} ml per cell. After microinjection cells were further cultivated in Eagle's medium supplemented with 2% foetal bovine serum, fixed and stained for PV-specific T and V antigen at different hours after microinjection. Intracellular T antigen formation was first detectable 6 h after cRNA transfer (Fig. 3). At this time about 6% of the recipient cells exhibited T antigen formation. After this time both the number of T antigen-positive cells as well as the intensity of T antigen-specific fluorescence per cell increased, reaching a maximum at 20–24 h. Thereafter both the proportion of T antigen-positive cells and the intensity of the T antigen-specific fluorescence decreased so that T antigen could not be detected 65 h after cRNA microinjection.

As shown in Table 1 the biological activity of the cRNA was destroyed by RNase treatment. Actinomycin D ($5 \mu\text{g ml}^{-1}$) inhibited T antigen formation in mouse cells either infected with wild-type PV (200 PFU per cell) or injected with PV DNA I (1 mg ml^{-1}), but did not even reduce cRNA-induced T antigen formation within the first 15 h. On microinjection neither total RNA nor polyadenylated RNA isolated from mouse cells induced T antigen formation.

PV cRNA preparation induced not only T but also V antigen (capsid protein) formation in microinjected secondary mouse cells. As shown in Fig. 3 the formation of V

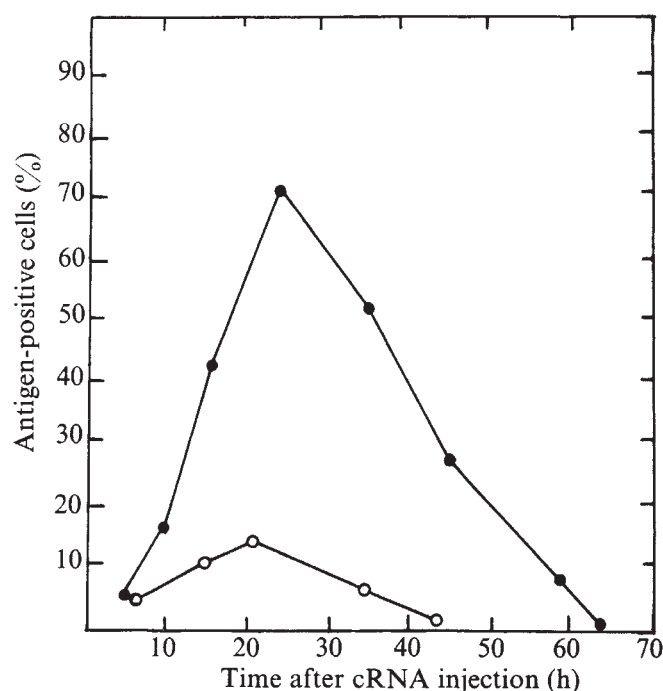
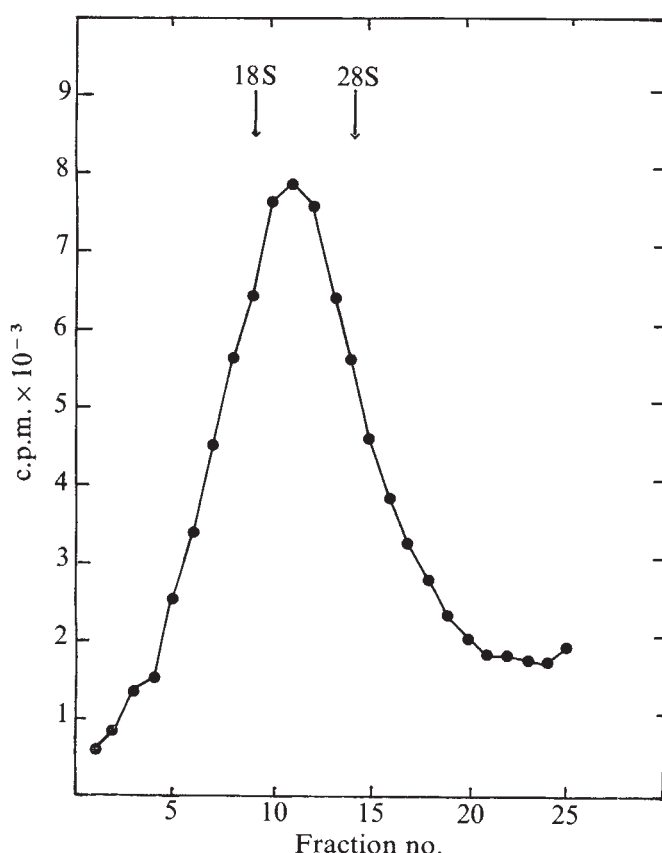


Fig. 3 Time course of T and V antigen formation in secondary mouse tissue culture cells. Secondary mouse cells grown on glass slides ($10 \times 50 \text{ mm}$, subdivided in squares of 1 mm^2) to confluence were used as recipient cells. Transfer of cRNA was carried out under a phase-contrast microscope ($\times 400$) by help of micro glass capillaries having a diameter at the tip of about $0.5 \mu\text{m}$. cRNA was dissolved in injection buffer ($0.048 \text{ M K}_2\text{HPO}_4$, $0.014 \text{ M NaH}_2\text{PO}_4$, $0.0045 \text{ M KH}_2\text{PO}_4$, $\text{pH } 7.2$) at a concentration of 0.5 mg ml^{-1} . At the time indicated cells were fixed (acetone-methanol 3:2 v/v, for 10 min at -20°C) and stained for T and V antigen by indirect immunofluorescence technique. Each time point is based on a count of 400 injected cells. ●, T antigen; ○, V antigen.

Fig. 2 Velocity sedimentation of PV cRNA in sucrose density gradient (7–27% sucrose w/v in high salt formaldehyde solution, 1.1 M HCHO , $0.09 \text{ M Na}_2\text{HPO}_4$, 0.001 M EDTA , $\text{pH } 7.7$). The gradient was centrifuged in a Spinco SW65 rotor at 49,000 r.p.m. for 3 h at 20°C . Before centrifugation PV cRNA was heated for 5 min at 80°C in low salt formaldehyde solution (1.1 M HCHO , $0.0045 \text{ M Na}_2\text{HPO}_4$, $0.0005 \text{ M NaH}_2\text{PO}_4$, 0.001 M EDTA , $\text{pH } 7.7$).



antigen on cRNA injection, although comparable in terms of time, was less efficient than the T antigen synthesis.

Our results demonstrate that the *in vitro* synthesis of PV cRNA, consisting of RNA complementary to both early and late viral DNA strand, induces PV-specific T and V antigen formation on microinjection into secondary mouse

Table 1 Polyoma virus T antigen formation in mouse cells

Injection of	Intranuclear T antigen formation (%) [*]
PV cRNA (0.5 mg ml^{-1})	42
PV cRNA (0.5 mg ml^{-1}) in actinomycin D-treated cells	43
PV DNA I (1 mg ml^{-1}) in actinomycin D-treated cells [†]	0
PV DNA I (0.01 mg ml^{-1}) [‡] + mouse cell RNA [§] (1 mg ml^{-1}) in actinomycin D-treated cells [†]	0
PV cRNA (0.5 mg ml^{-1}), RNase-treated ⁴	0
PV cRNA (0.5 mg ml^{-1}), alkaline-treated ⁴	0
Mouse cell RNA [§]	0

^{*}Each measurement is based on a count of 400 injected cells. Cells were fixed and stained 15 h after injection.

[†]Actinomycin D was added to a final concentration of $5 \mu\text{g per ml}$ medium 30 min before microinjection of nucleic acids. Cells remained in this medium during and after injection procedure until fixation.

[‡]Without actinomycin D this DNA concentration induces T antigen synthesis in 100% of injection cells.

[§]Total cellular RNA extracted from mouse kidney cells⁴.

tissue culture cells. The fact that the cRNA preparation was, in addition to DNase treatment, further purified by Cs_2SO_4 equilibrium centrifugation indicates that the virus-specific reactions are not due to a small contamination of viral DNA. This is also supported by the observation that cRNA-induced T antigen formation is sensitive to RNase treatment and that actinomycin D prevented T antigen synthesis in PV DNA-injected cell but not the cRNA-induced T antigen formation. These data support the hypothesis that PV-specific T antigen is a virus-coded but not a cell-coded protein. Our results do not exclude the possibility, however, that T antigen is a protein consisting of multiple subunits, in part virus and in part cell coded, or that the cRNA translation product modifies a constitutive cellular compound which then reacts immunologically as T antigen.

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The S value of SVDV isolates (UK, France 1/73 and 2862) and CB₅ isolates (Faulkner and 8068) measured in this way were similar to each other and to polio virus, indicating that the S value of polio virus (160S)⁸ can also be assigned to CB₅ and SVDV. Similar mixtures of purified viruses were used to compare the buoyant densities of SVDV and CB₅ in caesium chloride gradients ($\rho = 1.29\text{--}1.42\text{ g cm}^{-3}$) in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.6 by centrifuging at 75,000 g overnight at 20 °C (20,000 r.p.m., MSE 59108 rotor). All the SVDV isolates, CB₅ (8068) and polio virus came to equilibrium at a density of $1.34\text{--}1.35\text{ g cm}^{-3}$ whereas the prototype CB₅ (Faulkner) had the slightly higher buoyant density of $1.35\text{--}1.36\text{ g cm}^{-3}$. Buoyant density measurements have been used as a criterion for classifying mammalian picornaviruses and the values obtained here agree with those reported for other enteroviruses⁹. The slightly higher value obtained for prototype CB₅ is interesting and may

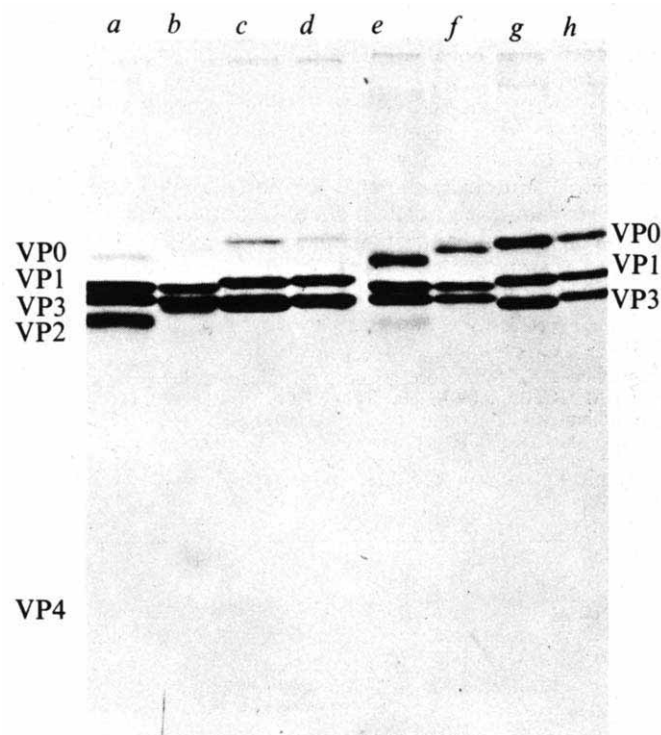


Fig. 1 Autoradiograph of a polyacrylamide gel slab of the polypeptides of ^{14}C -labelled full and empty particles of SVDV and CB₅ virus. Full particles of: a, SVDV (UK); b, SVDV (HK 71); c, SVDV (France 1/73); d, CB₅ (8068); e, empty particles of SVDV (UK); f, SVDV (HK 71); g, SVDV (France 1/73); h, CB₅ (8068). The viruses were grown in IBRS-2 cell monolayers¹⁵ labelled with ^{14}C -amino-acids ($5\text{--}10\text{ }\mu\text{Ci ml}^{-1}$, $45\text{ }\mu\text{Ci per m atom carbon}$, Radiochemical Centre) in 20 ml Earle's saline, 1.5 h after high multiplicity infection. They were purified from this medium after overnight incubation at 37 °C¹⁶. The 18-ml sucrose gradients (15–45% (w/v) sucrose in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.6 at 25 °C) were centrifuged at 80,000g for 2.25 h at 10 °C (30,000 r.p.m. MSE SW.25). The peaks of virus or empty particles were pooled, diluted with 0.1 M NaCl, 0.05 M Tris-HCl containing 75 μg bovine plasma albumin and protein precipitated with 2 volumes of acetone at $-20\text{ }^{\circ}\text{C}$ overnight. The precipitate was deposited by centrifugation, dried, resuspended in disruption buffer (0.0625 M Tris-HCl, pH 6.8 containing 0.5 M urea, 2% (w/v) SDS, 0.2% β -mercaptoethanol and 10% (v/v) glycerol) and analysed on high resolution acrylamide gel slabs¹⁷ using Tris-glycine buffer¹⁴. The main gel (12.5%) and the stacking gel (5%), modified to contain 0.5 M urea, were subjected to electrophoresis for 4–6 h at 100 V. The gel was stained with 0.25% (w/v) Coomassie brilliant blue in methanol-acetic acid (2:1), destained in methanol-acetic acid, dried under vacuum on to wettable Cellophane and autoradiographed with Kodak RP Royal X-Omat or Industrex X-ray film for 5–10 d.

Correlation of polypeptide composition with antigenic variation in the swine vesicular disease and coxsackie B₅ viruses

SINCE the observation by Graves¹ that swine vesicular disease virus (SVDV)² is neutralised by antiserum to coxsackie B₅ (CB₅) virus, an enterovirus infecting man, and observations³ that the swine virus can also infect man, there has been speculation about the relationship between the two viruses and the possible role of CB₅ virus in the aetiology of the swine disease⁴. Further work has shown that the two viruses can be differentiated by neutralisation tests and also that there has been considerable antigenic drift in the CB₅ viruses^{5,6}. RNA hybridisation experiments have corroborated these antigenic relationships; there is approximately 50% homology between the RNA of the UK isolate of SVDV and the RNA of the prototype (Faulkner) CB₅ and there is a similar level of annealing between prototype CB₅ RNA and the RNA of two recent isolates of CB₅ (8068 and 9954). These variations prompted an investigation into some of the other characteristics of SVDV and CB₅.

The sedimentation coefficient (S value) of several isolates of SVDV and CB₅ was determined and compared with that of poliovirus, the 'type' enterovirus⁷, by sedimenting mixtures of two viruses labelled with either ^{35}S -methionine or ^3H -amino acids (produced and purified as described in the figure legends) through 15–45% (w/v) sucrose gradients.

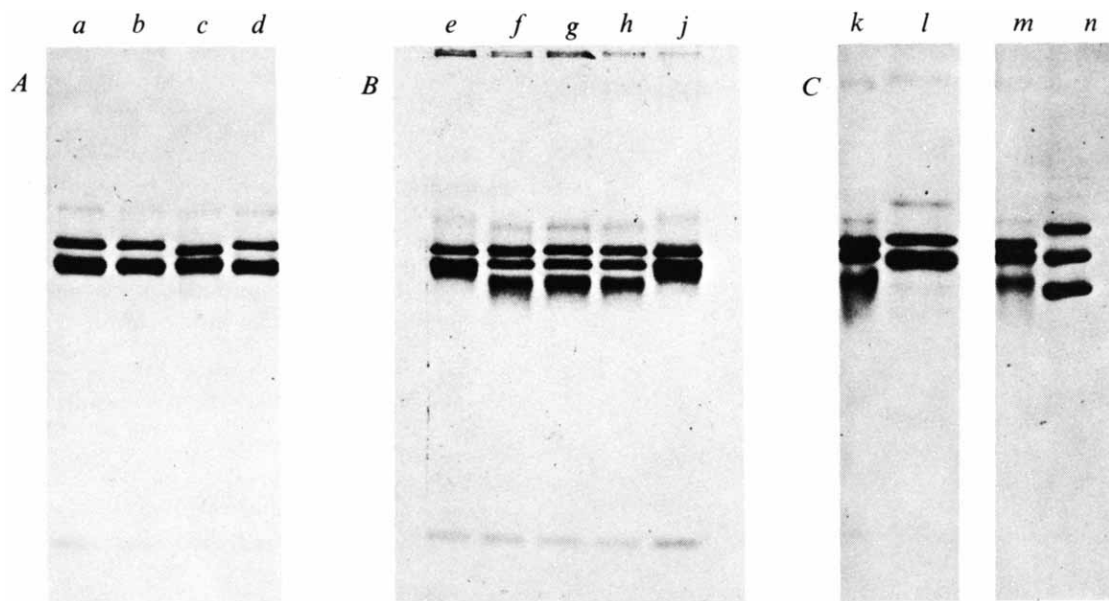


Fig. 2 Autoradiographs of polyacrylamide gel slabs of the polypeptides of several ^{35}S -methionine-labelled CB_5 and SVDV isolates. **A**, CB_5 isolates: *a*, 8068; *b*, 9954; *c*, Faulkner; *d*, HKB $_5$; **B**, SVDV isolates: *e*, Italy 1/66; *f*, UK; *g*, Austria 1973; *h*, Italy 1972; *j*, Hong Kong, 1971; **C**, SVDV isolates: *k*, UK; *l*, France 1/73; *m*, 2862; *n*, polio virus, type 1 (strain Mahoney). The viruses were grown, purified and prepared for electrophoresis as described in the legend to Fig. 1 but were labelled with ^{35}S -methionine ($2\text{--}5\ \mu\text{Ci ml}^{-1}$, $100\ \text{Ci mmol}^{-1}$, Radiochemical Centre) in 20 ml of methionine-free Eagle's medium. ^{35}S -polio virus was grown in HeLa cells in a similar way. The gels were subjected to electrophoresis and autoradiography as described in Fig. 1.

reflect some slight variation in the fine architecture of the virus particle of this strain.

The antigenic diversity between SVDV and CB_5 is presumably a reflection of some difference in the proteins of the virus particle. It seemed possible, therefore, that the pattern of polypeptides obtained by polyacrylamide gel electrophoresis of reduced and sodium dodecyl sulphate (SDS) disrupted virus particles might be distinct and perhaps diagnostic of a particular isolate. Comparison of the polypeptides of several purified isolates of SVDV and CB_5 by electrophoresis in 12.5% slab gels using a continuous buffer system at neutral pH (ref. 10), however, gave poorly resolved polypeptide patterns which could not be distinguished reproducibly. The patterns produced were similar to those published previously for selected strains of SVDV and CB_5 (refs 11 and 12) using a similar buffer system, but unlike that obtained by Delagneau *et al.*¹³ for the France 1/73 isolate of SVDV.

Well defined and reproducible differences in the mobility of the polypeptides were obtained, however, when a discontinuous Tris-glycine buffer system was used¹⁴ (Fig. 1). The most variable polypeptides in terms of mobility were VPO and VP2. Polypeptide VPO, the precursor of VP2 and VP4¹⁸, is found in empty particles and is also present in small amounts in the full particles of some picornaviruses^{19,20}. The mobility of polypeptide VP2, which was identified by its virtual absence from empty particles (compare Fig. 1*a* and *e*), also varied. This is clearly demonstrated by the patterns in Fig. 1*a–d*. Whereas the mobility of VP2 in SVDV (UK) (Fig. 1*a*) is clearly different from that of VP3, these polypeptides are not well separated in SVDV Hong Kong 1971 and France 1/73 (Fig. 1*b–c*). (Comparison of the separations obtained with the polypeptides of the full particles of SVDV (HK 71) and France 1/73 in Fig. 1*b* and *c*, with those obtained with the polypeptides of the corresponding empty particles in Fig. 1*f* and *g* shows that the lower intense band in the full particle patterns consists of two polypeptides VP2 and VP3.) The variation was reproducible and occurred whether the viruses were labelled with ^{14}C -amino acids or ^{35}S -methionine (compare Figs 1 and 2). Little variation was found in the mobility of VP4 (Fig. 2).

The patterns obtained for the four CB_5 viruses (Fig. 2*A*)

were clearly different from those of several of the SVDV isolates examined (Fig. 2*B* and *C*) as might have been expected from the clear cut differences found by neutralisation and immunodiffusion tests and by the RNA homology experiments^{5,6}. The patterns for the three recent CB_5 isolates (8068 and 9954 from Birmingham, England and HKB $_5$ from Hong Kong (Fig. 2*A*, *a*, *b* and *d*)) were identical but differed from that obtained for the Faulkner strain (Fig. 2*A*, *c*), again in agreement with the neutralisation and immunodiffusion data.

Of greater interest was the difference between isolates of SVDV which showed only small and probably insignificant differences in neutralisation tests²¹ and which had only been distinguished previously by the production of faint spur lines in immunodiffusion experiments^{3,5} and by rather less than complete annealing in RNA hybridisation experiments⁵. The patterns obtained with isolates from outbreaks in Italy in 1966 (Fig. 2*B*, *e*) and in Hong Kong in 1971 (Fig. 2*B*, *j*) were clearly different from those obtained with viruses isolated in England, Austria and Italy in the winter of 1972–73 (Fig. 2*B*, *f*, *g* and *h*). These data suggest that the viruses causing the 1966 and 1971 outbreaks and those causing the disease in Europe in 1972–73 had a different origin, and that the European outbreaks may have been caused by a virus from a common source.

An interesting observation made during the examination of the SVDV isolates was the difference between the polypeptide patterns of France 1/73 virus (Fig. 2*C*, *l*), isolated in the Bordeaux area in January 1973 and those of the viruses in other European countries at about that time (Fig. 2*B*, *f*, *g* and *h*). Immunodiffusion tests⁴ have shown that the France 1/73 isolate differed markedly from the other contemporary European isolates. It is also interesting that another isolate of SVDV (2862) (Fig. 2*C*, *m*) obtained from Annecy in the Haute Savoie area of eastern France at about the same time, gave a polypeptide pattern indistinguishable from that of the other European strains and also gave a line of identity with SVDV (UK) in immunodiffusion tests. Although the France 1/73 isolate of SVDV has a polypeptide pattern more like that of the recent CB_5 isolates, this need not imply that it is a CB_5 rather than an SVDV, but it emphasises the inherent variation in these viruses, the extent of which will only become clear after

examination of more strains. Further detailed biochemical analysis of this variation may help to establish more exactly the relationship of SVDV to the CB₅ viruses and give some insight into the evolution of the swine viruses.

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Synergistic effect of caffeine on the cytotoxicity of ultraviolet irradiation and of hydrocarbon epoxides in strains of Xeroderma pigmentosum

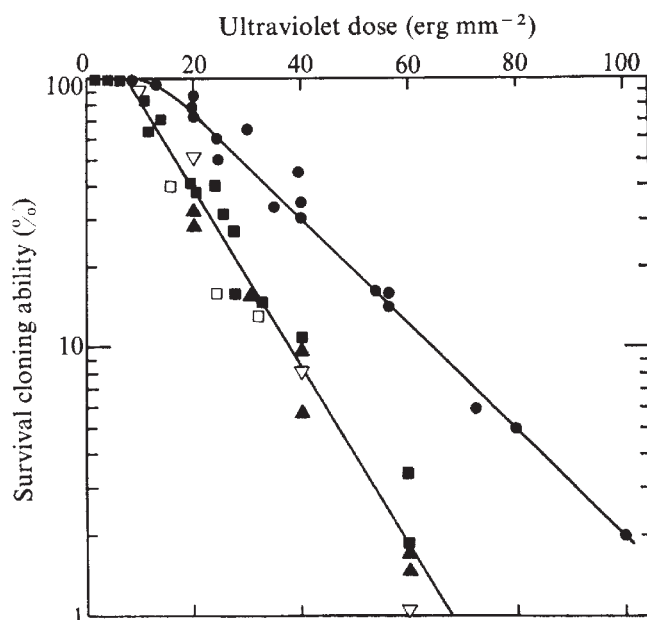
XERODERMA pigmentosum (XP) is an inherited human disease clinically characterised by abnormal pigmentation patterns and the development of multiple skin carcinoma on areas exposed to the Sun¹. Cells from most XP patients are defective in excision repair of ultraviolet-induced lesions in DNA^{1–3}, but there is a class of patients—XP variants—who have all the clinical manifestations of the disease but who, until recently, appeared to have normal DNA repair. Cleaver⁴ and others^{5,6} have shown that variants have normal rates of excision repair of damage caused by ultraviolet light as measured by ultraviolet-stimulated uptake of labelled thymidine or bromodeoxyuridine. Cleaver also reported that variants XP4BE (his patient 13) and XP13BE (his patient 14) have normal sensitivity to ultraviolet light as measured by survival of colony-forming ability⁴. His survival data, however, consisted of only two points for each strain. As Fig. 1 shows, we find that the percentage survival of the colony-forming ability of variants XP4BE and XP13BE as well as variant XP7TA and XP30RO is more sensitive to ultraviolet irradiation than that of normal cells. We also showed that the survival of the cloning ability of XP variants is more sensitive than normal to active derivatives of four aromatic amide carcinogens⁷ and to the K-region epoxides of three polycyclic hydrocarbons⁸. These two classes of carcinogens bind covalently to DNA^{9,10} and the bound adducts seem to be recognised by the same repair enzymes which function in the repair of ultraviolet-induced damage in these human cells^{7–11}.

Preliminary evidence, from alkaline sucrose gradient studies, that such XP variants suffer from a defect in their DNA synthesis following ultraviolet irradiation was reported¹² and

documented⁶ by Lehmann and his coworkers. They showed that of three variants tested after irradiation, all were abnormally slow in converting initially low molecular weight DNA into high molecular weight DNA similar in size to that produced in irradiated cells. Other investigators¹³ who reported that variant XP4BE (their patient WY-XP3), was normal in this gap-filling process had allowed 8 h for the process—too long to be able to distinguish the difference between XP variants and normal human cells. Lehmann *et al.*^{6,12} also reported that in the XP variants this gap-filling process was completely inhibited by 1.5 mM caffeine while that of normal cells was insensitive to caffeine. This process is thought to be involved in survival of cells which have replicated DNA containing unexcised thymine dimers which distort DNA and have introduced gaps in the newly synthesised DNA⁶. We, therefore, wanted to determine whether caffeine, in non-toxic or only slightly toxic concentrations, would affect the survival of XP variants but not normal human cells after exposure to ultraviolet irradiation or to carcinogens which form lesions in DNA which may be repaired by the same processes used for dimers produced by ultraviolet light.

First, we compared the sensitivity of the different strains to caffeine itself. Figure 2 shows that the colony-forming ability of two XP variants (XP4BE and XP13BE) is significantly more sensitive to caffeine than that of normal cells. To determine whether this increased sensitivity was characteristic only of XP variants, we tested the survival of the colony-forming ability of strains derived from three classical XP patients—each from a different complementation group¹, namely, XP12BE from group A, XP2BE from group C, and XP6BE from group D. The percentage survival of these strains in the presence of caffeine is also depicted in Fig. 2. The colony-forming ability of

Fig. 1 Percentage survival, as a function of ultraviolet dose, of the colony-forming ability of human skin fibroblasts (●) and that of four XP variant strains: ■, XP4BE; ▲, XP13BE; ▽, XP7TA; □, XP30RO. Survival of the cloning efficiency of irradiated cells divided by that of unirradiated controls is expressed as percentage. Cloning efficiencies of controls ranged from 15 to 35% for normal cells, from 10 to 25% for XP4BE and XP30RO and from 5 to 10% for XP7TA. The lower cloning efficiencies of this latter strain reflects the fact that it was available for testing only at a late passage number (> 30). The exponential portion of the curves were drawn by the least squares method. Each symbol represents the percentage survival averaged from a series of 8–12 replicate dishes of cells receiving the specified dose of ultraviolet irradiation. Variation in the number of colonies per dish was smaller than the size of symbol used. Procedures for cytotoxicity cloning experiments have been described elsewhere^{7,14}.



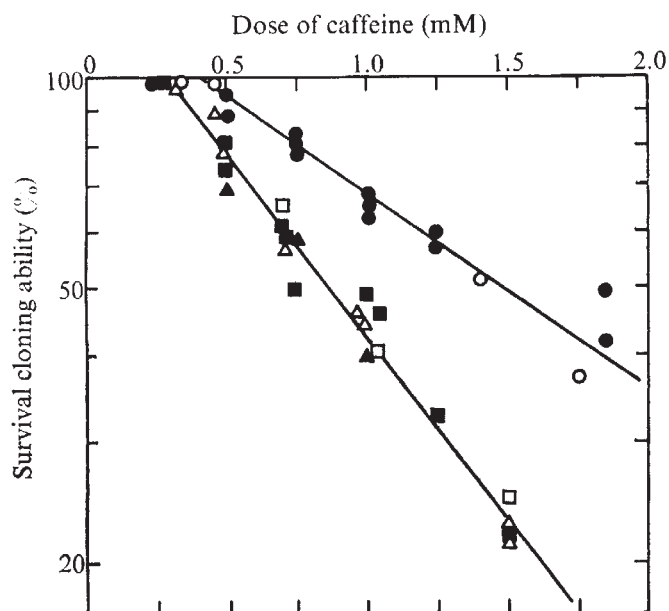


Fig. 2 Effect of caffeine on the cloning ability of normal cell (●), XP2BE (○), XP6BE (□), XP12BE (△), XP4BE (■), XP13BE (▲). From 250 to 6,000 cells in F10 medium (Ham's) containing 15% foetal bovine serum were plated in 60-mm dishes, allowed 8–10 h to attach and then fed medium containing the designated concentrations of caffeine three times weekly for 7–10 d and then caffeine-free medium until macroscopic colonies developed and could be stained. Survival of cloning ability was plotted as in Fig. 1.

XP12BE from complementation group A and XP6BE from group D proved to be just as sensitive to caffeine as the two XP variants, while the other classical XP strain resembled the normal cells in sensitivity to caffeine.

We then compared the effect of caffeine on the survival of the cloning ability of these strains after exposure to such DNA-damaging agents as ultraviolet irradiation or hydrocarbon epoxides. Cells were plated, exposed to the particular agent, and then left to form macroscopic colonies in the presence or absence of the specified concentrations of caffeine. The cytotoxic effect of each concentration of caffeine itself was determined from the untreated control dishes exposed to that particular dose. The effect of caffeine on the ultraviolet survival curves of normal cells, XP variants and classical XP strains is shown in

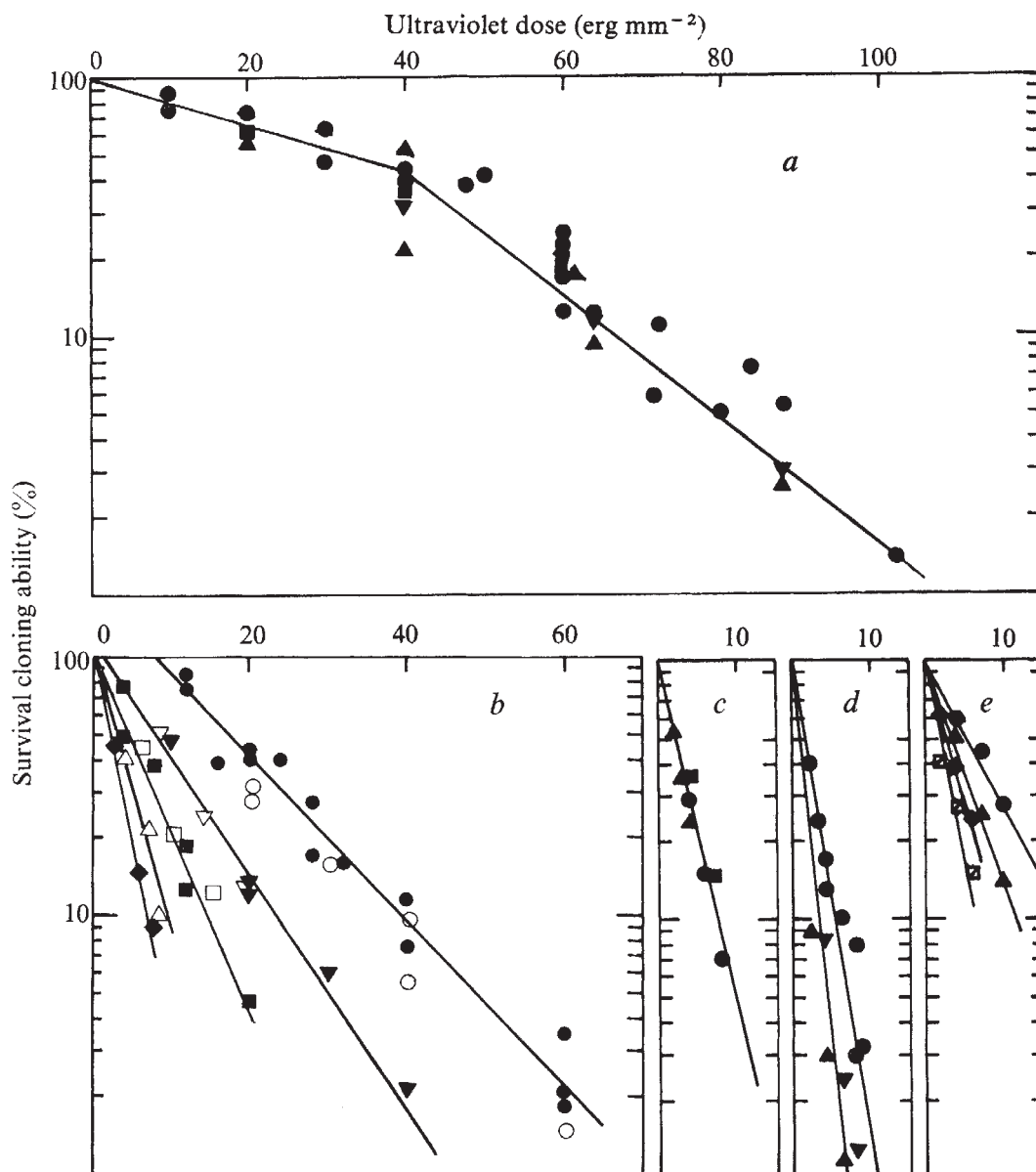


Fig. 3 Effect of caffeine on the cytotoxicity of ultraviolet irradiation in the various strains. Cells were plated, allowed to attach, irradiated as described previously⁷ with the specified doses of ultraviolet irradiation and fed medium containing the specified concentrations of caffeine three times weekly for 7–10 d and then caffeine-free medium until macroscopic clones developed. For each concentration of caffeine, the survival of the cloning abilities of the particular strains are plotted as percentage of cloning efficiency of its unirradiated control to correct for any killing by that particular concentration of caffeine. Cells were protected from light during all operations. *a*, Normal fibroblasts; *b*, XP4BE (closed symbols); and XP13BE (open symbols); *c*, XP6; *d*, XP12; *e*, XP2. Concentrations of caffeine (mM): ●, 0.0; ▼, 0.5; ■, 0.75; ▲, 1.0; diamond, 1.5; square and horizontal bar, 1.75.

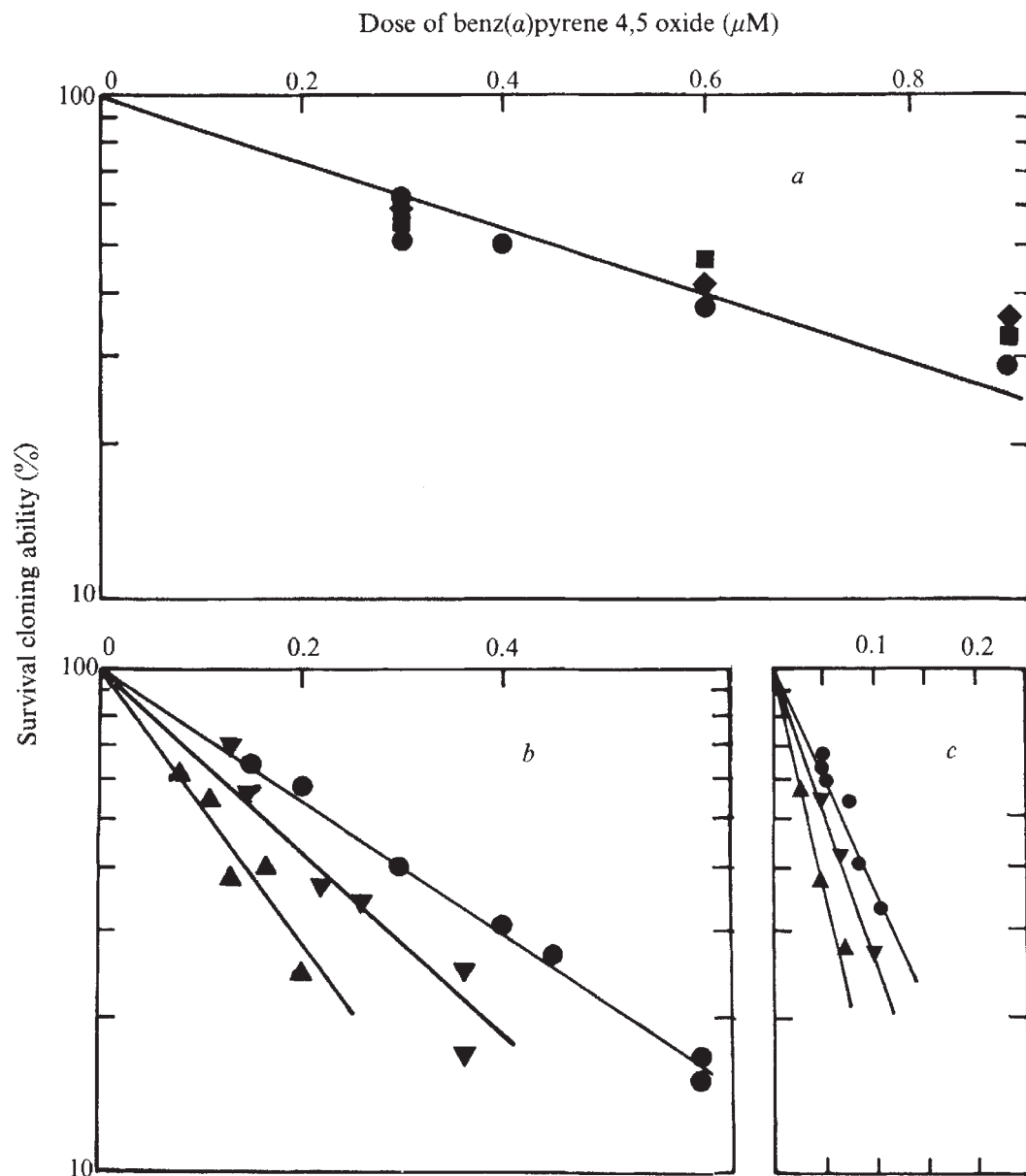


Fig. 3. Each survival curve was corrected for any cell killing caused by exposure to the caffeine alone. Therefore, an increased slope in a survival curve indicates that the presence of caffeine during the repair period has potentiated the killing effect of the ultraviolet exposure, that is, it has had a synergistic effect on the cytotoxicity of ultraviolet irradiation. The data show that even in a dose as low as 0.75 mM, caffeine increases the cytotoxicity of ultraviolet irradiation in the variant strains, XP4BE and XP13BE, and that this synergistic effect increases with increasing concentrations of caffeine. In contrast, caffeine, at comparable cytotoxic concentrations (compare Fig. 2), causes no measurable increase in the slope of the ultraviolet survival curve of the normal cells or XP6BE from group D and, at most, exerts a slight synergistic effect on the classical XP strains, XP12BE group A and XP2BE group C.

The rate of the gap-filling process in a classical XP from group A and from group C, as measured by physical means, is reported⁶ to be intermediate between that of normal cells and XP variants and to be intermediately sensitive to caffeine. This may account for the slight synergistic effect of caffeine observed in these biological studies. We find, however, no direct correlation between the sensitivity of a cell strain to caffeine and the ability of caffeine to exert a synergistic effect on ultraviolet cytotoxicity in that strain.

We have shown that ratios of the slopes of the survival curves

of XP variants and two classical XP strains to that of normal cells after exposure to hydrocarbon epoxides resemble those found for ultraviolet irradiation⁷. This suggests that these cells repair at least some of the DNA damage caused by the hydrocarbon treatment by processes similar to those used to repair ultraviolet damage. We therefore compared the effect of caffeine on the survival of such strains after exposure to the K-region epoxide of benz(a)pyrene (Fig. 4). It is evident that caffeine, even in cytotoxic doses, has no potentiating effect on the cytotoxicity of this compound in the normal cells; exerts a dose-dependent synergistic effect on the killing by the epoxide in the XP variant, and has an intermediate effect in the classical XP from group A. These results support the hypothesis that the cellular DNA repair processes involved in handling such DNA-damaging agents are similar to those used for ultraviolet induced lesions. We are examining these strains of human cells for the effect of caffeine on cell survival after exposure to other types of DNA-damaging agents, which induce ultraviolet-like repair, such as aromatic amide carcinogens.

Cleaver has shown that caffeine does not inhibit unscheduled DNA synthesis in HeLa cells¹⁵. Our survival curves for the normal fibroblasts support this conclusion. Since the XP variants have been shown by several methods to have normal excision repair⁴⁻⁶, one would not expect caffeine to inhibit excision in these strains. It seems to interfere with the gap-filling

Fig. 4. Effect of caffeine on the cytotoxicity of benz (a) pyrene 4,5 oxide in the various strains. Percentage survival of the cloning ability at each concentration of caffeine was plotted as in Fig. 3. Detailed procedures for the exposure of cells to freshly prepared anhydrous acetone solutions of the carcinogen have been described^{7,8}. a, normal fibroblasts; b, XP4BE; c, XP12. Symbols represent concentrations of caffeine as in Fig. 3.

process⁶ and it renders the cloning capacity of these cells more sensitive than normal to DNA-damaging agents. The mechanism by which caffeine causes the observed effects in the XP strains is unknown. But survival studies such as ours are important because they demonstrate that a defective repair process, previously identified by physical techniques, manifests itself directly in a reduction in cell survival. Our data with these XP variant strains provide evidence, in addition to that of Day¹⁶, for a relationship between defective DNA repair and the clinical manifestation of XP.

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Cyclic AMP-induced changes in protein synthesis in a cellular slime mould, *Polysphondylium pallidum*

In addition to acting as a chemotactic signal^{1,2}, cyclic AMP induces the formation of stalk cells from undifferentiated amoebae in the cellular slime mould *Dictyostelium*^{3,4}. The inductive action is specific for cyclic AMP, as AMP, ATP, ADP, GMP and GTP have no effect⁵. The induction also acts on *Polysphondylium pallidum*, a related species, even though cyclic AMP is not chemotactic for this species^{5,6}.

There are two hypotheses at present for the general mechanism of cell differentiation in these organisms: Sussman⁷ has proposed that a succession of new genes becomes active and that the resulting succession of newly synthesised enzymes causes the morphological changes of cell differentiation. This agrees with the known effects of cyclic AMP on transcription of new genes, both in higher eukaryotes⁸⁻¹⁰ and in bacteria^{11,12}. Wright and colleagues^{13,14} argue that the causes of visible development are primarily a succession of changing substrate concentrations, interacting with a relatively stable complement of enzymes. Newly synthesised proteins are not the primary "critical variables" by this theory. Rosness and Wright¹⁵ have suggested that cyclic AMP might act on cell differentiation by directly affecting the conversion of glycogen to cellulose in stalk cells and spores.

In this paper, acrylamide gel electrophoresis and auto-

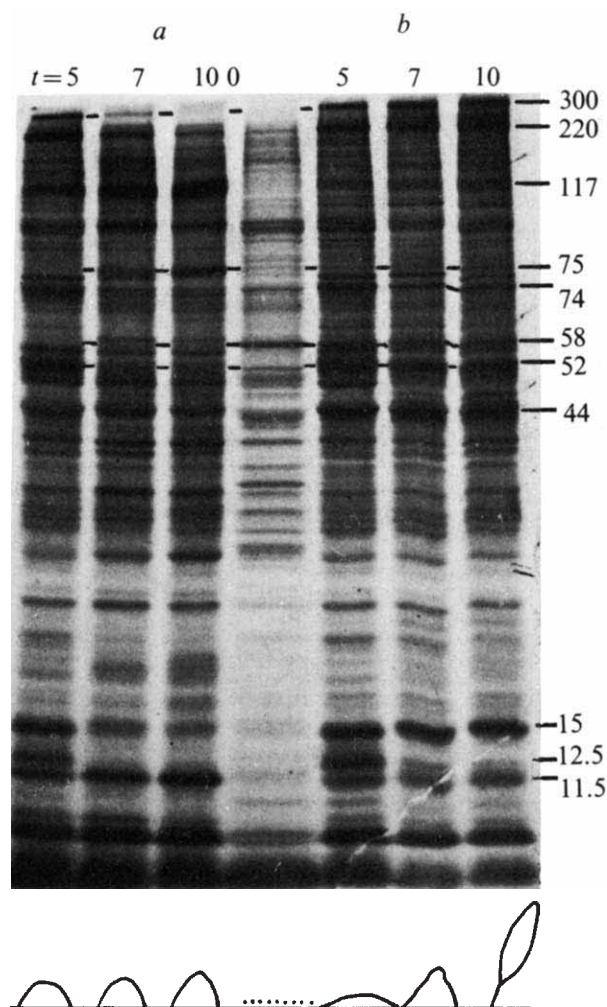


Fig. 1 Autoradiographs of ¹⁴C-labelled proteins of developing *P. pallidum* cells. Times are hours between dawn and the start of the labelling hour. Cyclic AMP was added to one set of cups at 2 h (a). b, Buffer treated. The stage of development is indicated by diagrams beneath each column. Aggregation mounds eventually became heaps of mixed amoebae and stalk cells in the cyclic AMP-treated cells. Band numbers $\times 10^3$ indicate approximate molecular weights as determined by a series of known proteins. The sample for column 1 contained 6,650 c.p.m., the others 15,000 c.p.m. Culture and labelling: Cells of *P. pallidum* strain PN501 (ATCC number 26659) were grown in complete darkness in shaker flasks on *E. coli* B/r (ref. 17) until the bacteria were gone and the amoebae began to clump. The cultures were centrifuged without cooling and the pellet resuspended in 0.017 M phosphate buffer, pH 6.0. The cells were dispensed on to a dialysis membrane stretched over one end of a 0.5-inch diameter plastic cylinder (a 'cup'), at a density of 5×10^6 cells per cup (corresponding to 240 μ g protein per cup and to 4.5×10^4 cells mm^{-2}). These operations were carried out under a sodium-vapour darkroom safelight emitting light of 589 nm, a wavelength to which this strain is very slightly sensitive¹⁸. Development was initiated by exposing the cups to fluorescent light of intensity 138 lx. At the same time the cups were inverted over a layer of charcoal, which absorbed volatile inhibitors of development, and the dialysis membrane was covered with a buffer-soaked filter disk to prevent drying. To label proteins, the filter disk was replaced with a Millipore filter saturated with 25 μ l buffer or with buffer with 5×10^{-3} M cyclic AMP added, plus 10 μ C per pad of *L*-¹⁴C sodium acetate. The total acetate concentration was 7×10^{-3} M. Cells were collected after one hour of labelling by brushing them into 100 μ l sample buffer¹⁹ added to the cup. Samples were stored at -90°C . Electrophoresis: Samples of dissolved cells were thawed, then placed in boiling water for 1 min to dissolve proteins. In general, samples were diluted so that 25 μ l had 10,000-50,000 c.p.m. TCA-precipitable material; this amount was used for electrophoresis. Where practical the samples run on a single gel were diluted to equal c.p.m. Discontinuous SDS electrophoresis in 10% polyacrylamide slabs was by a modification of the procedure of Laemmli^{19,20}. After electrophoresis, the gels were stained with Coomassie blue, dried and autoradiographed using Kodak X-ray film RP/R54, developed with Diafine developer.

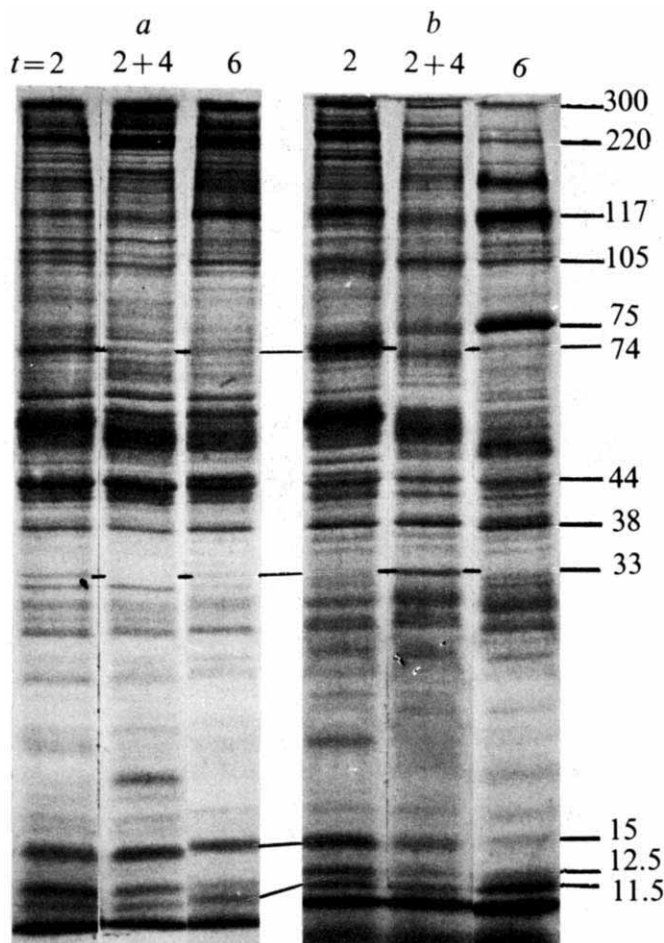


Fig. 2 Autoradiographs of pulse-chase experiment. Here cyclic AMP was added to the experimental set at dawn ($t = 0$). Cups labelled at $t = 2$ h were collected either at the end of the 1-h labelling period (first and fourth columns) or after a 4-h chase period (second and fifth columns). To chase, the Millipore disk containing labelled acetate at 1×10^{-3} M was replaced by a filter paper disk of cold acetate at 7×10^{-3} M. Comparison of changes in individual band intensities in columns 1 and 2 with those in columns 4 and 5 indicates the degree to which cyclic AMP affects proteolysis. Band 33 increases in intensity during the chase period in cyclic AMP-treated cells, and may represent a subunit created by induced proteolysis of a larger protein. *a*, Buffer treated; *b*, cyclic AMP-treated.

radiography were used to compare synthesis of specific proteins in normally developing and in cyclic AMP-treated cells. The results indicate that induction of stalk cells by cyclic AMP necessarily involves alterations in net synthesis of several proteins.

Dark-grown cells of *P. pallidum* were dispensed on to dialysis membranes stretched over small cups. Development was initiated by exposing the cups to light. Two hours after dawn development was in mid-aggregation, and some of the cups were exposed to 5 mM cyclic AMP. To detect protein synthesis, cells were labelled with ^{14}C -acetate and collected at intervals from time zero (dawn) to 10 h (the early sorogen stage in the untreated cups). Control cups were inspected on the following day for presence or absence of heaps of mature stalk cells. The cell samples were used for measurement of trichloroacetic acid (TCA)-precipitable material and for sodium dodecyl sulphate (SDS)-acrylamide electrophoresis.

An immediate effect of cyclic AMP exposure was a temporary decrease in the rate of precursor incorporation into TCA-precipitable material by approximately 30%.

In addition cyclic AMP induced differences in labelling between normally developing and treated cells in about 10 of the approximately 55 resolvable bands shown in Fig. 1. Roughly half of the changes consist of the disappearance of a band in the cyclic AMP-treated cells, whereas the same band remains steady in the normally developing controls (for example, numbers 300, 220, 74, 58 and 15). In three cases, a band becomes denser in the cyclic AMP-treated cells and remains steady or fades in the control cells (number 117, 75 and 11.5). In at least one case (12.5) a band which changes during normal development changes in a similar fashion in cyclic AMP-treated cells. A large majority of bands are steady in both wild type and cyclic AMP.

In every case except one, the changes are in development-correlated bands—that is, bands which change in one way or another during normal development between time zero when the lights are turned on and 10 h. These observations indicate that the effect of cyclic AMP is not to affect proteolysis or protein synthesis in general, but is highly specific.

To distinguish between changed synthesis and changed degradation as causes of altered protein net synthesis, cells were pulse labelled with ^{14}C -acetate for 1 h, then chased with cold acetate for 4 h. The results (Fig. 2) indicate that fading of certain bands following cyclic AMP treatment is accelerated (numbers 300, 220, 15 and 12.5), whereas others remain relatively constant (numbers 38, 44 and 105). This indicates that cyclic AMP induces specific proteolysis. In the case of two bands which increase in intensity following cyclic AMP treatment (numbers 11.5 and 75) no proteolysis is detectable with or without cyclic AMP, which suggests that cyclic AMP induces new synthesis of these proteins.

Bonner³ has reported that stalk cell induction by cyclic AMP occurred even in the presence of cycloheximide, which (if so) would argue against the notion that new protein synthesis is essential for stalk cell induction. When cycloheximide ($200 \mu\text{g ml}^{-1}$) was added to cells of *P. pallidum* at the same time as cyclic AMP, total protein synthesis dropped to 21% of control, and synthesis of most proteins, including all of those affected by cyclic AMP, was greatly reduced. These amoebae did not differentiate to stalk cells. This contradiction of Bonner's finding remains unexplained.

In other circumstances in which cyclic AMP does not induce stalk cells the specific band changes do not occur. Cells kept in constant darkness, for example, failed to aggregate and did not respond to cyclic AMP addition by either specific band changes or subsequent stalk cell differentiation. Similarly, cells which had embarked on formation of microcysts and exhibited a corresponding set of protein changes different from those of aggregation, also showed no response to cyclic AMP.

The fact that precursor incorporation into protein bands shows specific increases or specific decreases following cyclic AMP treatment, together with the fact that these changes are always correlated with the induction of stalk cells and do not occur when stalk cells are not induced, argues strongly that cyclic AMP exerts its inductive effect on cell differentiation by affecting synthesis and breakdown of particular proteins. To this extent the results support the hypothesis of Sussman. Note, however, that while these changes in protein synthesis are occurring, the cells are still amoeboid. The morphological changes of increased vacuolisation and formation of cell wall polysaccharides which result in actual stalk cells¹⁶ do not occur until 10–20 h later. It remains possible that the temporally immediate causes of these morphological changes are fluctuations in substrate concentrations, as proposed by Wright. A general theory of development might include elements from both hypotheses.

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Actin polymerisation induced by spectrin

EUKARYOTIC cells are thought to contain a framework of fibrillar proteins—or 'cytoskeleton'—which is a basis for their shapes and movements. To understand this structure it is necessary to know both the properties of the component proteins and the way they are linked together. Actin and spectrin are two such structural proteins: actin is familiar for its part in muscle contraction, but is present partly as microfilaments^{1,2} throughout the cytoplasm of most cells; spectrin is the major protein of red blood cell membranes and makes up a network on the cytoplasmic surface^{3,4}.

We were led to study the interaction of spectrin and actin by the observation that endogenous membrane-associated actin copurified with the spectrin of human erythrocytes. Spectrin is extracted together with small amounts of protein of lower molecular weight from erythrocyte ghosts in media of low ionic strength. When purified by column chromatography on Sephadex G-200, the spectrin emerges at the void volume and most of the other protein elutes later. Spectrin so isolated is pure, as judged by gel electrophoresis in the presence of SDS, except that a protein which migrates at the same position as muscle actin is always present. This corresponds to membrane component 5 in the nomenclature of Fairbanks *et al.*⁵, and evidence that it is like actin in other respects has been mentioned⁶. For the experiments described below spectrin was prepared by extraction of washed ghosts with water for 1 h at 37 °C; it was purified by chromatography on a column (2.2 × 50 cm) of Sephadex G-200, and concentrated when necessary by vacuum dialysis. Rabbit muscle actin was prepared according to the method of Spudich and Watt⁸. At low ionic strength muscle G-actin remains in a monomeric state (molecular weight 42,000), and does not polymerise, so that on a Sephadex G-200 column in these conditions it should readily separate from spectrin. We observed, however, that nearly all the protein in an actin-spectrin mixture emerged in a single peak which showed both components on SDS-acrylamide gels.

To establish whether actin had bound to spectrin to form aggregates, or had been induced to polymerise into filaments, we measured the viscosity of actin-spectrin mixtures. In this low ionic strength solution, actin or spectrin alone had low and constant viscosities, but on mixing, the viscosity rose sharply in a manner characteristic

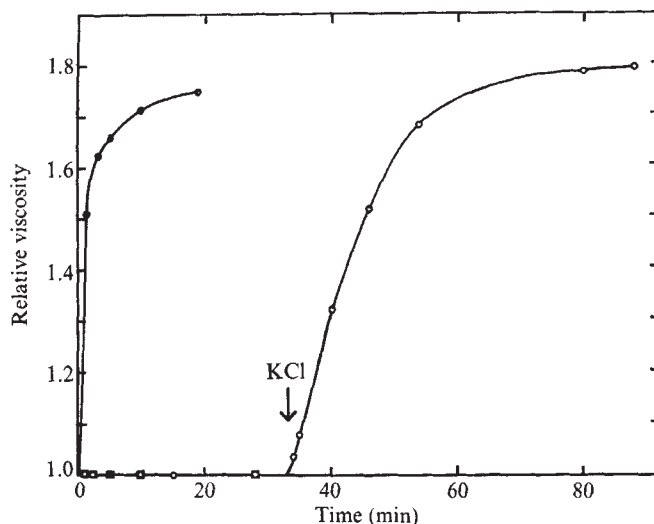


Fig. 1 Increase in relative viscosity of an actin-spectrin mixture. The viscosities of protein solutions containing actin alone (○), actin and the haemoglobin-containing fraction (■), and spectrin with actin (●), were observed with time. Spectrin in this case was an unchromatographed fresh extract of human erythrocyte ghosts containing 0.4 g l⁻¹ spectrin; G-actin from rabbit skeletal muscle was used at 0.6 g l⁻¹. 2-ml aliquots for viscosity in S buffer (see Table 1) were introduced into an Ostwald viscometer maintained at 30 ± 0.1 °C, and the flow time for the sample was measured at intervals. The polymerisation of actin alone was induced by addition of KCl to a concentration of 0.1 M at the point indicated by the arrow.

of the formation of long polymers (Fig. 1). The same results were obtained when EDTA was present to eliminate calcium and magnesium ions. The final viscosity reached was similar to that shown by actin alone in the presence of 0.1 M KCl; if KCl was added to the actin-spectrin mixture no further increase was observed.

Evidence of specific interaction was also obtained by electron microscopy. Negatively-stained preparations of either the actin or the spectrin showed only small globular subunits (with spectrin we find that aggregation depends critically on the pH of the medium⁹); but in the mixture there were abundant filaments (Fig. 2). These seemed to have the form of actin filaments but were predominantly associated in parallel bundles. The polymers could be collected by centrifugation (100,000g for 1 h) and gave a clear pellet in conditions in which neither actin nor spectrin alone gave an appreciable residue (Table 1). Analysis of this pellet on SDS gels showed that it contained a preponderance of actin but some spectrin remained even after repeated washing.

Table 1 Formation of sedimentable material in actin-spectrin mixture

Protein	Buffer	Sedimented protein (μg)
G-actin (250 μg)	D	37
G-actin (250 μg)	D+0.1 M NaCl	225
G-actin (250 μg)	S	25
Spectrin (1,040 μg)	S	50
Actin (250 μg)+spectrin (1,040 μg)	S	213
Actin (250 μg)+spectrin (400 μg)	S	63

Solutions were made up in a total volume of 0.5 ml at 4 °C and then warmed to 25 °C for 10 min. They were then cooled to 4 °C and centrifuged at 100,000g for 1 h. The sediments were carefully rinsed and then suspended in 0.5 ml of 1% sodium dodecyl sulphate. Protein values were estimated by the Lowry method using BSA as a standard. D buffer; 2 mM Tris-HCl, 0.2 mM CaCl₂, 0.2 mM dithiothreitol, 0.2 mM ATP (pH 7.4). S buffer; 10 mM sodium phosphate, 0.2 mM dithiothreitol, 0.1 mM CaCl₂, 0.2 mM ATP (pH 8.2).

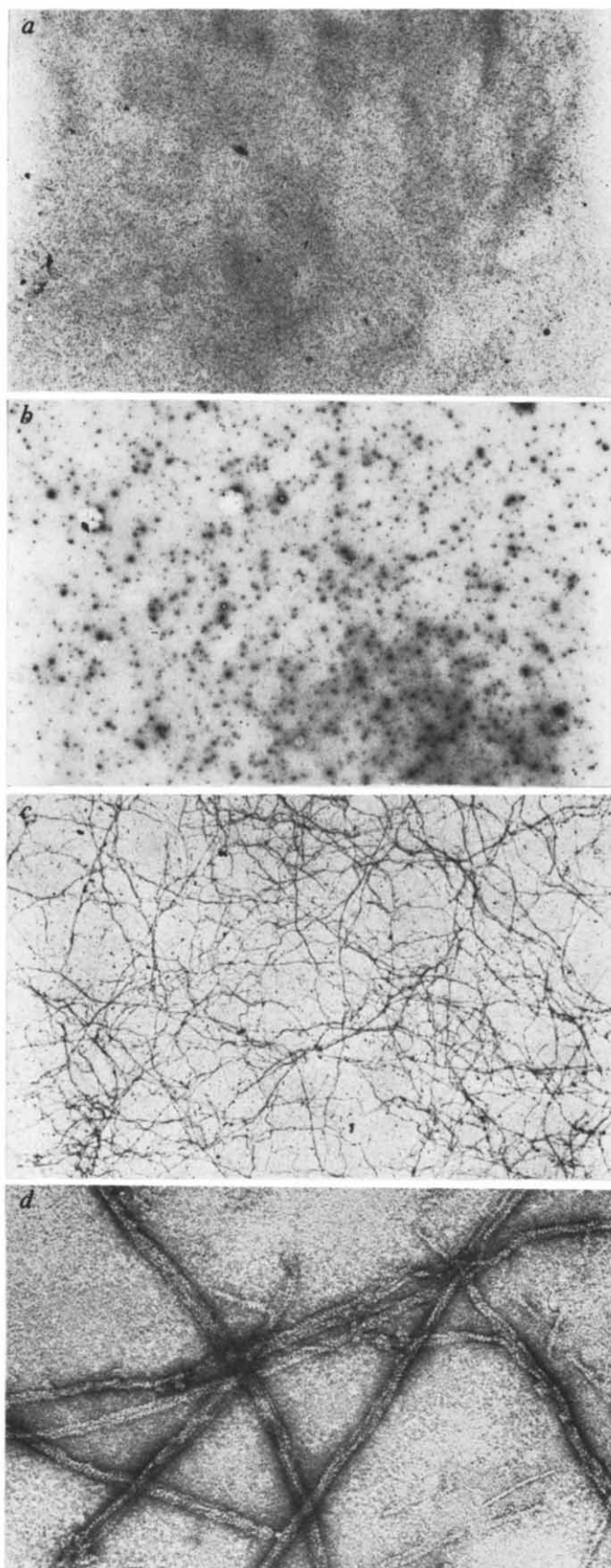


Fig. 2 Generation of filaments in an actin-spectrin mixture. Protein solutions were in S buffer and at similar concentrations to those described in Table 1. Samples were diluted to 0.1 g l^{-1} protein, applied to carbon-coated grids, stained with uranyl acetate and examined in a Phillips 200 electron microscope. *a*, Actin alone; *b*, spectrin alone; *c* and *d*, actin-spectrin mixture. Magnification: *a-c*, $\times 8,250$; *d*, $\times 90,000$.

It therefore seems that spectrin can promote the polymerisation of muscle actin, as judged by the elution profile of the actin in gel filtration, the rapid increase in the viscosity of mixtures, the transformation of the actin into a rapidly sedimenting form, and the appearance of filaments in the electron microscope. This effect could not be reproduced with fractions from the gel filtration column that did not contain spectrin, with haemoglobin or serum albumin in place of spectrin, or with the solution remaining after heating the spectrin to 100°C .

We believe it is significant that we have been able to observe the interaction between spectrin and G-actin only when the spectrin preparation is fresh, and when it has been extracted from freshly drawn and not stored (for example outdated blood-bank) blood.

After completion of the experiments described above, a report by Tilney and Detmers¹⁰ appeared, which also gave evidence of an interaction between spectrin and actin. In contrast to our results at low ionic strength, these authors observed a diminution in the maximum value of the viscosity generated by the salt-induced polymerisation of G-actin when spectrin was present. These data may not be incompatible with our findings, in consequence of the different ionic strength, the lower spectrin concentration and the possibly different age of the preparations.

These differences dictate caution in extending the results of any experiments of this kind to the conditions existing in the cell. As in other systems involving fibrous proteins¹¹, spectrin and actin probably give rise to polymorphic aggregates, the nature of which is finely governed by the milieu. It is clear, however, that they do interact and that this interaction may be of great importance, particularly since proteins similar to spectrin are not restricted to the mammalian erythrocyte, and have been found in invertebrate sperm¹², macrophages¹³, and sea urchins¹⁴.

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Arrhenius parameters of phototransients in *Halobacterium halobium* in physiological conditions

Halobacterium halobium is indigenous to warm saline pools exposed to bright sunlight. Strains of this bacterium synthesise a purple pigment in the cell membrane (the 'purple membrane'). A single protein, bacteriorhodopsin, is responsible for this characteristic colour, possessing a broad band in the visible spectrum with a maximum at 570 nm (refs 1-3). Pigmented

cells utilise solar radiation absorbed by bacteriorhodopsin to create a transmembrane proton gradient which serves as an energy source for ATP synthesis^{1,2}. Most information about the mechanism of operation of this light-driven proton pump has been obtained from spectrophotometric studies of suspensions of purple membrane fragments⁶⁻¹⁰. We report here a continuation of studies initiated in this laboratory using modulation excitation¹¹ and conventional flash photometry^{12,13}, in which we use the latter technique to observe transient spectral changes in aqueous suspensions of intact cells at physiological temperatures and salinity (basal salt solution).

The principal difficulty in spectroscopic studies of cell suspensions is the intense scattering that obtains in this system. This is particularly acute in flash photometry since long light paths are used generally to monitor the absorption of transients. We have overcome this difficulty in two ways. First, operating our analysing lamp at maximum intensity and using maximum amplification in our recording electronics¹³ we could work with relatively short path lengths (5 mm). Second, we reduced scattering by the presence of 40% (w/v) sucrose in the basal salt medium. By this means the refractive index of the medium is increased to approximately that of the cell and scattering becomes negligible. Sucrose was chosen for this purpose since carbohydrates are not metabolised by *H. halobium*¹⁴ and therefore would not be expected to penetrate the cell membrane. We found also that the presence of this concentration of sucrose does not alter the spectrum of the phototransients and in addition the coupled formation of pH gradient and synthesis of ATP under continuous illumination proceeds normally.

Transient spectra recorded from flash-photolysed cells in basal salt were essentially identical to those recorded for

Fig. 1 Representative appearance and decay profiles of transients in flash-photolysed *H. halobium* cells in basal salt. The photolysing flash was filtered through a Corning 3-69 cutoff filter ($\lambda > 520$ nm). Percentage transmission increases on going from top to bottom. *a-c*, 5 °C; *d-f*, 30 °C. *a*, 410-nm transient: appearance, 0.5 ms per division; decay, 20 ms per division. *b*, 570-nm transient: appearance, 0.5 ms per division; decay, 20 ms per division. *c*, 660-nm transient: appearance and decay, 20 ms per division. *d*, 410-nm transient: appearance, 0.1 ms per division; decay, 5 ms per division. *e*, 570-nm transient: decay at 0.1 and 5 ms per division (due to light scattered from the flash reaching the photomultiplier (uppermost trace) grow-in at this wavelength could not be resolved). *f*, 660-nm appearance and decay, 5 ms per division.

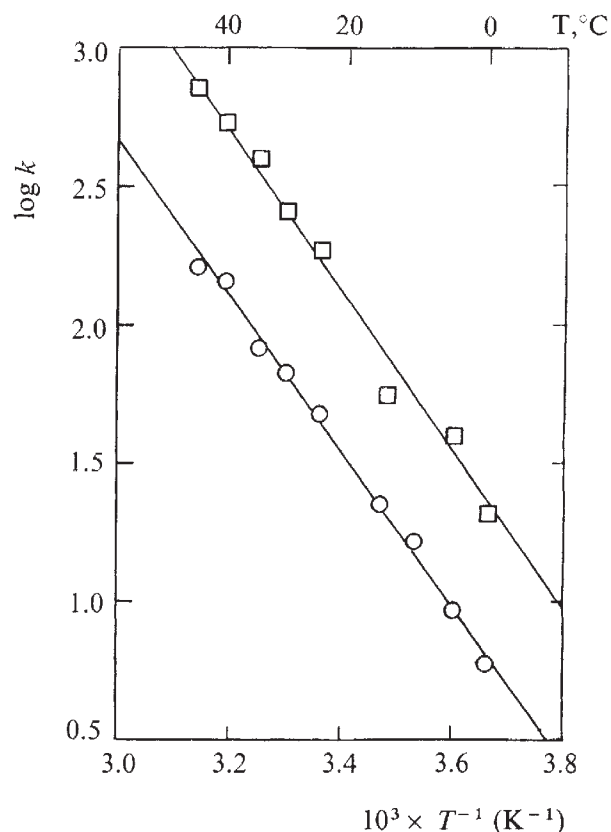
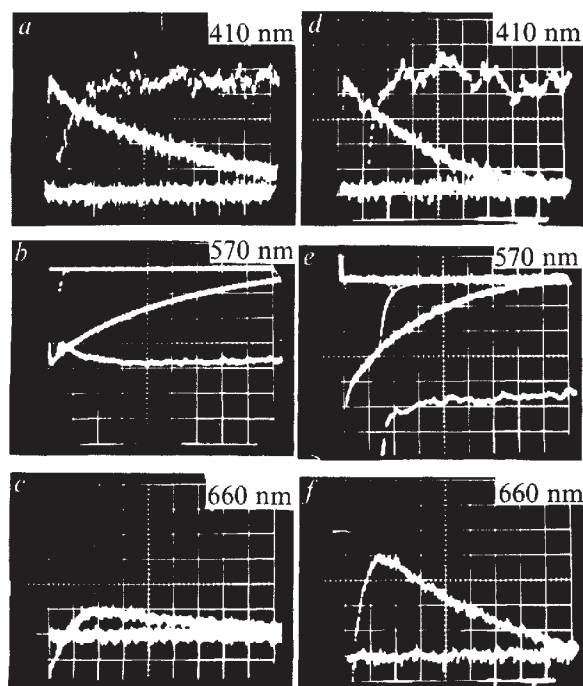


Fig. 2 Arrhenius plot of first-order rate constants for decay of 410-nm phototransients in whole cells (○) and purple membrane fragments (□). Cells were suspended in basal salt, purple membrane fragments were suspended in water.

aqueous suspensions of purple membrane fragments^{12,13}, exhibiting two chromophores with maxima at 410 and 660 nm respectively, and concomitant depletion centred at the absorption maximum of the initial state chromophore at 570 nm. An isosbestic point was found at 458 nm. Neither of the transient chromophores represent primary photochemical species since relatively long grow-in times were observed (Fig. 1). A similar long grow-in of the 570-nm depletion is also apparent. This is a characteristic of purple membrane suspensions, and evidence was obtained that this phenomenon is a consequence of an overlap from a shorter lived phototransient (maximum about 530 nm) which is the precursor of the 410-nm species^{12,13}.

Cell suspensions were studied in the temperature range 0–45 °C, about 37 °C being optimum for cell growth. At each temperature decay of the phototransients and the concomitant reappearance of the ground state chromophore obeyed first order kinetics with equal half lives. The maximum intensities of the 410 and 570-nm transients were relatively insensitive to temperature, while that for the 660-nm transient decreased with decreasing temperature, becoming undetectable at 0 °C. Grow-in and decay times of all three phototransients increased with decreasing temperature. The first-order rate constants for their decay conform to an Arrhenius plot (Fig. 2) with energy of activation $E_a = 13$ kcalorie mol⁻¹ and entropy of activation $S_a = -7$ e.u. Results obtained with purple membrane suspensions in water are also presented in Fig. 2, and yield similar activation parameters ($E_a = 13$ kcalorie mol⁻¹, $S_a = -4$ e.u.). At the same temperatures phototransients in intact cells exhibited longer half lives than those in purple membrane suspensions, possibly a consequence of the difference in suspension medium (Fig. 2 legend).

As found for purple membrane suspensions¹³, flash photometry using polarising filters showed all three phototransients in intact cells to be polarised. Only small decreases in dichroic

ratios were observed during the transient lifetimes showing the bacteriorhodopsin molecule to be essentially immobilised within its membrane.

The transients we have observed clearly relate to the final step of the proton-pumping cycle, although the question as to whether they occupy a common or parallel pathway is under discussion (W.V.S., R. Korenstein and S.R.C., manuscript in preparation). Based on recent Raman studies^{15,16}, a convincing argument has been presented which identifies the 410-nm and 570-nm chromophores as representing, respectively, free and protonated Schiff bases¹⁶. The coupled decay of the 410-nm transient and regeneration of the 570-nm chromophore represents a last step in the cycle. This step involves no breaking of bonds or molecular rearrangement and may be expected to be diffusion-controlled, that is dependent on the rate at which protons can diffuse through the membrane (or through the peripheral regions of the protein molecule itself) to the site of the chromophore. The small entropy of activation that we obtain agrees with that which may be estimated from the data of Lanyi for Brownian movement within membrane vesicles from the related *H. cutirubrum* (about -9 e.u.)¹⁷ and is therefore consistent with this proton diffusion model. The activation energy that we obtain is higher than Lanyi's (3-4 kcalorie mol⁻¹) and may indicate that lateral proton diffusion in *H. halobium* is associated with a particle which has a larger effective size than that for rotational diffusion of the perylene molecule used as a probe in the *H. cutirubrum* study. (Alternatively, diffusion within the protein may be the limiting factor.)

Several similarities between bacteriorhodopsin and vertebrate rhodopsin have been identified^{1,4,7}. Our results, however, point to two significant differences. First, whereas rapid rotation of the pigment within its membrane is possible in vertebrate rhodopsin^{18,19}, bacteriorhodopsin is immobilised within its membrane. The second difference relates to proton uptake. In vertebrate rhodopsin, uptake of one proton per rhodopsin molecule is associated with the thermal relaxation of metarhodopsin I → metarhodopsin II (refs 20 and 21). First-order treatment of the spectral intensities associated with these intermediates yields $E_a = 31$ kcalorie mol⁻¹ and $S_a = +55$ e.u. (ref. 22). Analysis of the data on proton uptake²¹ yields consistent values of $E_a = 27$ kcalorie mol⁻¹ and $S_a = +42$ e.u. The magnitude of these activation parameters for proton uptake in vertebrate rhodopsin therefore shows significant molecular rearrangement in the rate determining step (release of phospholipids has also been identified at this stage²²). In contrast, proton uptake in bacteriorhodopsin is quite different involving, as we have shown, small activation parameters and therefore little molecular rearrangement in its rate determining step.

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Nanosecond flash photolysis of rhodopsin

THIS note presents results from a neodymium laser flash spectroscopy study of bovine rod outer segments (ROS), the only chromophore of which is rhodopsin. Previously unreported transients are interpreted in terms of a charge-transfer excited state which gives rise to radicals and/or triplets.

The change in the absorption spectra of visual pigments such as rhodopsin after irradiation is a much studied process following the original work of Wald¹. These studies have used predominantly conventional spectrophotometry, or conventional flash photolysis with micro- or millisecond resolution times. To study the processes occurring after the pigment absorbs a photon (rhodopsin → prelumirhodopsin (PLR) → lumirhodopsin (LR)) low temperatures were used.

Recent reports of work at room temperature or above using nanosecond resolution times or less were limited to a narrow range of wavelength detection. Cone² reported transient absorption changes at 510 nm and 580 nm, and concluded that PLR decays to LR with a half life of 170 ± 30 ns and LR decays to metarhodopsin I (MRI) with a half life of 50 ± 20 μ s. Busch *et al.*³ studied rhodopsin with a mode-locked laser and monitored transient absorption changes at 560 nm. They estimated that PLR is formed in 6 ps or less and decays with a lifetime of about 30 ns. Rosenfeld *et al.*⁴, using an N₂ laser to excite rhodopsin in the β band (330 nm), were able to measure transient absorption changes at wavelengths between 575 and 650 nm. They excited outside the main α absorption band of rhodopsin and consequently required the use of high optical densities of rhodopsin at wavelengths corresponding to the α band (4.0 at 498 nm). They estimated a PLR lifetime of ~ 50 ns and a maximum near 580 nm.

We used a Nd laser flash apparatus⁵. Most of our results concern the use of the 530 nm excitation line obtained by frequency doubling the laser output. A few results using 265-nm and 353-nm excitations are also included. Spectral measurements were made over the wavelength range 320-610 nm. The ROS (ref. 6) were solubilised with 3% Emulphogen in Tris buffer at pH 8.2. For the various samples of ROS used the ratio A_{278}/A_{500} was in the range 2.0-2.2.

Our observations are best considered in terms of typical oscillograms at varying monitoring wavelengths. Fig. 1 shows such oscillograms at 570 nm, 500 nm, 450 nm and 350 nm. At 570 nm we observed (Fig. 1a) an immediate increase in absorption. This transient absorption decays with a half life of 125 ± 25 ns giving a final increase in transmission compared with the original absorbance. Laser excitation at 353 nm and at 265 nm leads to oscillograms similar to that at 570 nm. Monitoring at 500 nm, with 530-nm excitation (Fig. 1b), we observed a rapid decrease followed by an increase in absorption with a half life of ~ 125 ns. At 450 nm (Fig. 1c), an initial fast increase in absorption is followed by a further increase in absorption with a half life of ~ 125 ns. We estimate the lifetime of this secondary transient to be between five and several tens of μ s. At 350 nm (Fig. 1d and e) a short lived species with half life of ~ 125 ns, is followed by a longer lived transient with a half life of ~ 5 μ s. In order to test for the

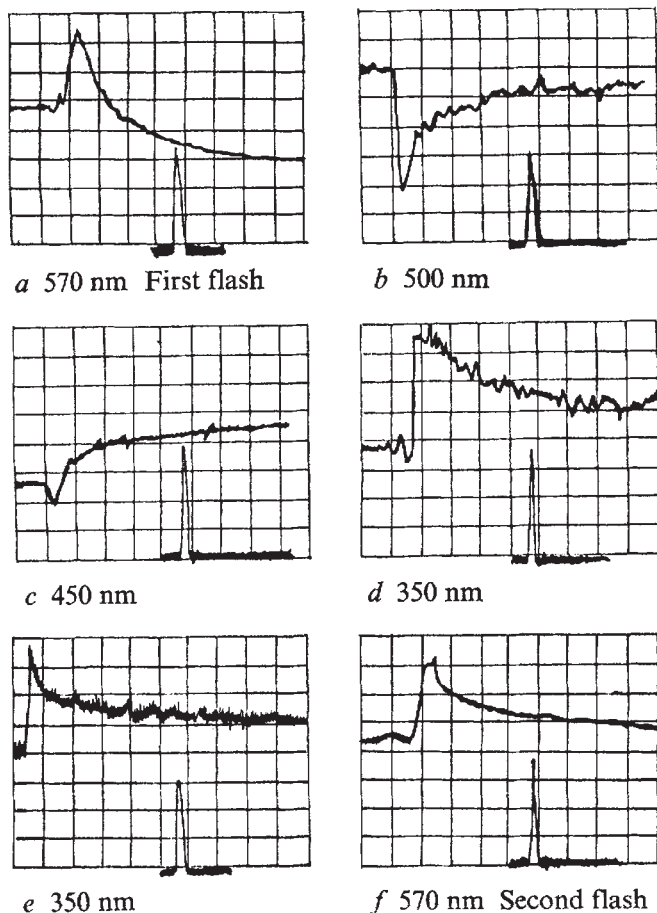


Fig. 1 Oscilloscope traces of absorption changes following 530-nm laser excitation of ROS in 3% Emulphogen. Horizontal displacements; time (1 large division equals 100 ns except for *e* where it equals 500 ns). Displacements vertically upward; decreasing transmission (1 large division equals 5.88, 1.96, 6.55, 1.05, 1.08 and 5.88% change in transmission for *a-f* respectively). A fresh solution was used in each case except for *f*. The ROS concentration for *a* and *f* was ten times that for *b-e*. The traces at the bottom of each photo arise from the photodiode which monitors the laser flash intensity from flash to flash. The initial increase in transmission in *b* and *c*, over within 50 ns, is due to scattered laser light.

possibility of two photon effects, the laser intensity was varied over a sixfold range. Since only linear variations in absorbance with light intensity were obtained, two photon effects are not occurring.

Figure 2a shows the difference spectra obtained 50 and 800 ns after the beginning of the flash. We have assumed that the transient absorption band at 550 nm is symmetrical and have used the fact that the transient spectrum after 50 ns is isosbestic with rhodopsin at 537 and 479 nm. Even if our assumption is incorrect, the position of the various maxima we observe is unaffected, only the relative intensity of the bands is changed. Figure 2b shows the transient spectra after these corrections. Table 1 shows our estimated extinction coefficients at these maxima using a value of $41,000 \text{ M}^{-1} \text{ cm}^{-1}$ as the extinction coefficient of rhodopsin⁷.

After the ROS have been illuminated, similar transients to those above are observed over the wavelength range 320–400 nm; Fig. 1f shows a typical oscillogram at 570 nm. The unbleached ROS show an initial increase in absorption (Fig. 1a). Bleached ROS, that is, after 1 or more flashes or after standing in normal room lighting for several minutes, show an initial increase in absorption but no subsequent decrease compared with the original absorption (Fig. 1f). The transients we observe from bleached ROS may arise from pararhodopsin⁸.

The usual structural interpretation of the spectral changes after the initial absorption of light by visual pigments such as

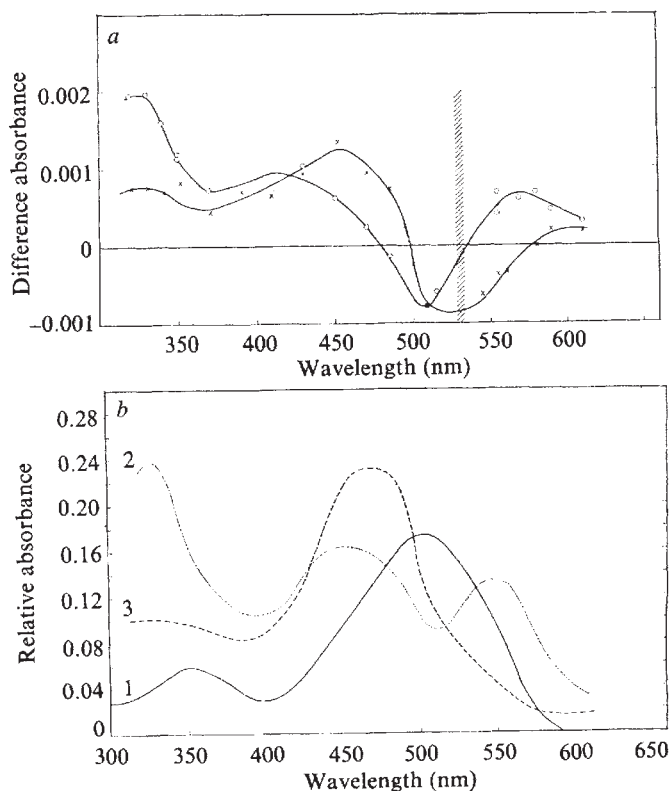
Table 1 Maximum molar extinction coefficients at 293 K		
Species	Wavelength (nm)	Extinction coefficient ($\text{M}^{-1} \text{ cm}^{-1}$)
Rhodopsin	500	41,000*
First transient	550 ± 10	31,500 ± 3,000
	450 ± 10	38,800 ± 4,000
	330 ± 10	56,100 ± 5,500
Second transient	470 ± 10	54,900 ± 5,500

*See ref. 7.

rhodopsin, is that following the initial 11-*cis* to *trans* isomerisation of retinal (to form PLR), there are various conformational changes of the protein, opsin, the consecutive transients formed being called LR, MRI and metarhodopsin II, and so on.

The earliest transient from ROS (curve 2 in Fig. 2b) has a maximum absorption on the long wavelength side of the rhodopsin absorption, but also shows two additional bands at about 450 and 330 nm. A long wavelength (550 nm) absorption is well known at low temperatures¹ and was called PLR. The second formed transient spectrum (curve 3 in Figure 2b) shows a single maximum absorption on the short wavelength side of rhodopsin and seems to correspond to the LR (or MRI) absorption at low temperatures¹. Thus, while some of our results might seem to be in agreement with the previous low temperature studies in terms of PLR and LR (or MRI) the extra peaks observed immediately after the laser flash do not seem to fit in with the accepted scheme. Yoshizawa^{9,10} has observed an intermediate absorbing at ~430 nm (hypso-rhodopsin) on irradiation of rhodopsin at liquid helium temperature. This is converted into PLR on raising the temperature or on absorption of light of wavelength below 430 nm. We do not believe that the peak we observe at $450 \pm 10 \text{ nm}$ is the same species since it is formed and decays at the same rate as the peaks at 550 and 330 nm.

Fig. 2 *a*, Absorption changes following 530-nm laser excitation of ROS in 3% Emulphogen. ○, ~50 ns and ×, 800 ns after the beginning of the laser flash. *b*, Corrected absorption spectra of first (2) and second (3) transients. The absorption spectrum of the original ROS is also shown (1).



We believe that tryptophan is involved with retinal in the chromophore. The results of Lewis and Spoonhower¹¹ using resonance Raman spectroscopy imply the existence of a strong complex between retinal and a tryptophan residue in rhodopsin. Shirane¹² claims from electron spin resonance evidence that the excitation energy absorbed in the range 450–600 nm by frog rhodopsin results in the formation of the triplet state of tryptophan. Various transient absorption spectra from tryptophan in aqueous solution have been reported by Santus and Grossweiner¹³. A short lived transient absorption with a maximum at 460 nm was assigned to the triplet state of tryptophan. We have previously¹⁴ reported a transient absorption of 11-*cis* retinal at 445 nm which we assign to the retinal triplet state. The first transient absorption we now observe on flash photolysis of ROS includes a maximum at 450 ± 10 nm. Santus and Grossweiner¹³ assign a transient absorption at 340 nm and a weaker transient at about 600 nm with a shoulder at 550 nm to the radical cation of L-tryptophan and 1-methyl-L-tryptophan. The wavelengths of the maxima of these two transient absorptions are close to the maxima at 330 ± 10 and 550 ± 10 nm we observe as part of the first transient obtained on flash photolysis of ROS. The second transient (Fig. 2b) shows only one maximum, at 470 ± 10 nm. Santus and Grossweiner¹³ reported an absorption at 485 nm after flash irradiation of L-tryptophan at pH 5.4 at 25 °C which they assign to the neutral radical formed by deprotonation of the radical cation of tryptophan.

Charge transfer involving an electron donor-acceptor situation in which the complexes are stable in the ground state are well known. They give transient spectra, showing features of both radical cations and anions and of excited states of the individual components of the complex¹⁵. Excitation at any wavelength where the charge transfer complex absorbs may lead to a single species and the lifetime of the individual bands associated with this species would be identical. Furthermore, Ishigami *et al.*¹⁶ and Mendelsohn¹⁷ have both suggested that a charge transfer complex between indole rings and retinal exists and is responsible for the major absorption band in the region 500–600 nm for such complexes.

We suggest that an excited state of the charge transfer complex rhodopsin may be the primary intermediate involved in the vision process. The products of this excited state may be radicals and/or triplets rather than only conformational changes of opsin. The primary intermediate (first transient) bears the characteristic absorption bands of tryptophan, retinal radical ions and triplet states. Since excited states are short lived even at very low temperatures, it is not surprising that the first transient we observe does not appear in the low temperature spectrum reported by previous workers after low intensity irradiation of rhodopsin.

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Equatorial X-ray reflections and cross arm movement in skeletal muscle

WHEN resting skeletal muscle is excited or goes into rigor, there is a characteristic change in the ratio of the intensities of the two principal equatorial reflections, I_{11}/I_{10} . This has been interpreted simply as reflecting movement or transfer of mass in the radial direction from the thick to thin filaments^{1,2}. Other, higher orders, have, however, been observed^{1,3}, and a more detailed interpretation is possible, which should eliminate some of the ambiguity that results from using only two reflections. Since there is considerable evidence that the myosin filament is actually three or four stranded^{4–6}, rather than two stranded as originally proposed, reinterpretation is necessary. Further, studies of X-ray diagrams from muscle in the presence of ATP analogues suggest that at least in some cases, equatorial intensity ratios intermediate between rigor and relaxed do not arise from a mixture of those states, but rather from a state with different structural properties^{7–9}.

The number of observed equatorial reflections is very limited (compare Table 1). This, plus the difficulty of determining the phases, means that a completely unambiguous answer is not possible; however, various models of the packing of the actin and myosin within the hexagonal filament lattice, can be described and the mass projected on to a unit cell in the equatorial plane. From such models, predicted phases and relative intensities can be calculated and then compared with experimental results to eliminate some possibilities. Five such models are shown in Fig. 1 and the predicted intensities are given in Table 1, together with experimentally measured intensities from a frog sartorius muscle (*Rana pipiens*) at a sarcomere length of 2.2 μ m.

The difficulty of deciding between different models solely on the basis of I_{11}/I_{10} can be seen by comparing the last four columns in Table 1. The four models all give reasonable 'relaxed' ratios. The higher order reflections provide more information and allow certain distinctions to be made. Models II and III are unsatisfactory in predicting appreciable intensities for the higher orders. Any model with short cross arms will probably have a fall off in intensity much sharper than observed experimentally. Models IV and V, with longer arms, are better in predicting higher orders, though refinement of the models would clearly be necessary for a precise fit. It would, however, be easier to fit the experimental equatorial data by using models with cross arms projecting to a radius of 180–200 Å. Most importantly, such models with longer arms will fit axial diffraction data better. Cross arm packing similar to either model IV or V will fit experimental intensity measurements of the first-layer line from frog muscle, assuming that the thick filaments are either three or four stranded (R. W. L. and G. H. Cohen, in preparation). Thus, both the axial and equatorial

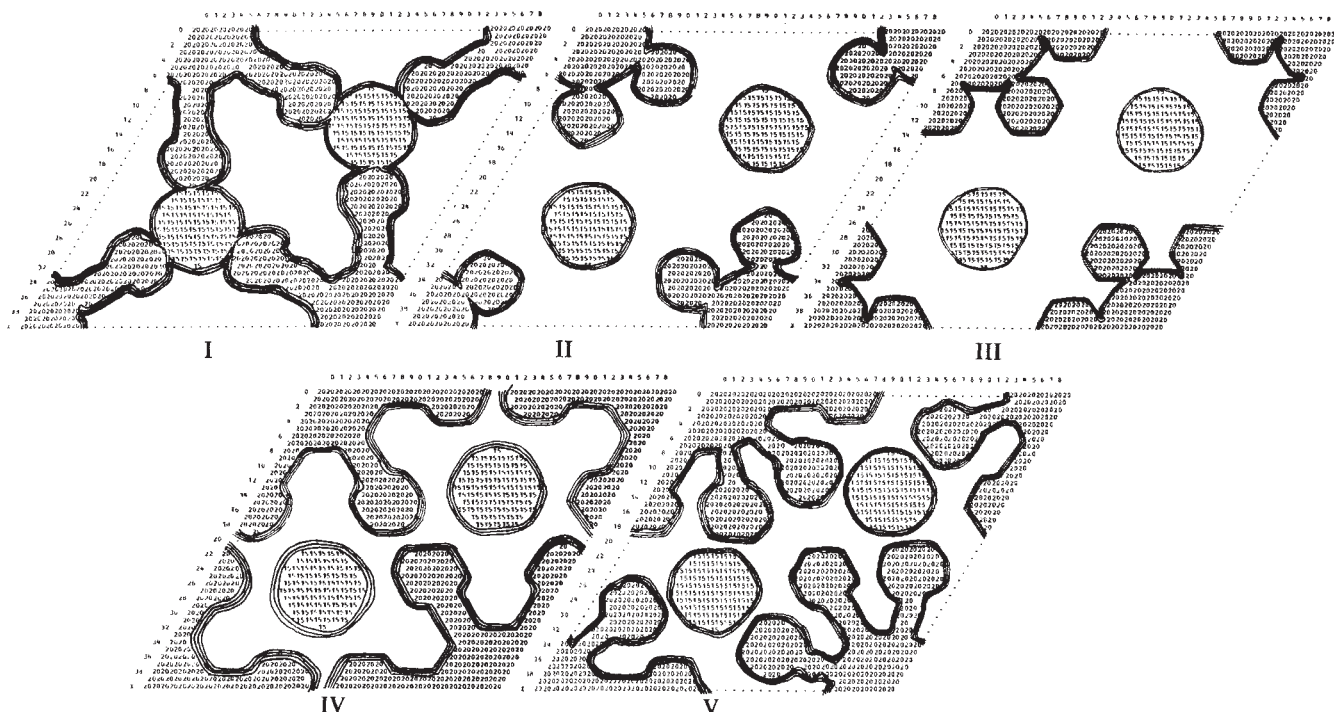


Fig. 1 Five models of the packing of myosin cross arms within the unit cell of the hexagonal lattice of filaments, projected on to the equatorial plane (equivalent to looking along the fibre axis). The backbone of the thick filament is located at the corners of the unit cell. The projection of the two actin filaments per unit cell are the circles at the trigonal points. The actin filament is assumed to project to a circle of radius 40 Å, whereas the core of the myosin filament is 60 Å radius. The myosin cross arms assume that the two subunits lie side by side, to fuse to one, almost circular, head. The dimensions, and relative mass within a myosin 'molecule' approximate reported values¹⁰. The actin-myosin mass ratio has a value of 0.25, which is close to what one expects in the region of overlap of a frog sartorius muscle with a sarcomere length of 2.2 μm. In models II and III, the globular cross arms of myosin project to a radius of 130 Å, whereas in models I, IV, and V the arms project to a radius of 180 Å. The side of the unit cell is 400 Å. Model V is a projection expected from a three-stranded thick filament, with one of the cross arms in the *10* plane (at an azimuthal angle of 0°). Models III and IV have cross arms at an azimuthal angle of 0°, while I and II form azimuthal angles of 30°. Model I gives an I_{11}/I_{10} that is typical of rigor muscle, while models II, III, IV, and V all give ratios typical of resting muscle.

diffraction patterns can be fitted assuming that the cross arms project to a radius of the order of 180–200 Å. In insect flight muscle it also seems that models in which the cross arms extend far from the thick filament¹² can fit the relaxed pattern.

Thus the difference between the I_{11}/I_{10} from models IV and I is most provocative. The only difference between the two models is that in model IV the myosin cross arm forms an azimuthal angle of 0° with the *10* plane, while in model I, the angle is 30° (that is, the cross arm is in the *11* plane). This allows for the strong possibility that the change in I_{11}/I_{10} when a muscle is excited, arises mainly from azimuthal variations in

the configuration within the equatorial projection, with little radial movement outwards from the thick filament (shown schematically in Fig. 2). This suggests that excitation of the muscle causes azimuthal disordering of the myosin cross arm with respect to the helical packing of the thick filament backbone, with simultaneous weakening of the relaxed layer-line pattern. Those cross arms which attach to actin can be further twisted, azimuthally and axially (that is, in a plane perpendicular to the page), to produce the slewed actin-myosin rigor state reported by Moore *et al.*¹³. This would explain many of the changes seen in X-ray diagrams on excitation, and generate

Table 1 Relative equatorial intensities*

Model	I	II	III	IV	V	Experimental
Azimuthal angle	30°	30°	0°	0°	0°	
Radius	long	short	short	long	long	
Rotational symmetry	6	6	6	6	9	
Reflection						
<i>10</i>	100(0)	100(0)	100(0)	100(0)	100(0)	100
<i>11</i>	324(0)	47(0)	33(0)	30(0)	25(0)	44
<i>20</i>	46(π)	7(π)	3(π)	36(0)	5(0)	9
<i>12, 21</i>	61(0)	3(π)	10(π)	13(π)	36(0)	9
<i>30</i>	15(π)	0(0)	18(0)	150(0)	35(0)	12
<i>22</i>	33(0)	2(0)	1(π)	11(0)	19(1.4)	20
<i>13, 31</i>	30(π)	0(0)	0(0)	21(π)	84(2.1)	

* Intensities are calculated from the amplitudes, F , assuming that the experimentally observed pattern is equivalent to a rotation of the unit cell around the fibre axis. Thus $I_{hk} \propto (F_{hk})^2 / \sqrt{(h^2 + k^2 + hk)}$. The predicted phases, in rad, are given in parentheses. They are very model dependent for higher orders.

Amplitudes and phases were calculated from the models in Fig. 1 using the fast Fourier program CHAFF described by Bantz and Zwick¹⁴. The *22, 13, 31* reflections could not be resolved experimentally.

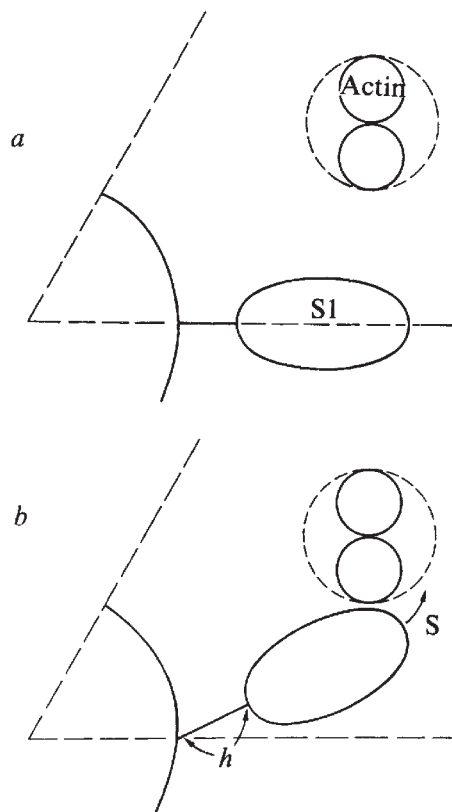


Fig. 2 Schematic representation in the equatorial plane of possible two-dimensional organisations of actin and myosin molecules. *a*, A section of the unit cell in Fig. 1. A single subfragment 1 (S1) moiety, containing one actin-binding site and one ATPase site, projects from the thick filament backbone to a radius of 210 Å, centred in the 10 plane for demonstration. Two actin monomers are shown at the trigonal point, in an orientation favouring creation of an actin-myosin link. *b*, When the muscle is excited, the S1 moiety can be displaced azimuthally from its rest position. (Note that S1 in the 'rest' state is actually fluctuating about the single rest state shown in *a*. The azimuthal displacement may simply represent its immobilisation by binding to actin.) This causes a change in I_{11}/I_{10} . On binding to actin, further movement may occur (arrow (S)), as the moiety slews round the actin within the equatorial plane, as well as in the axial direction (perpendicular to the page). Flexibility is allowed in the entire subfragment 2 region, between the two arrows, *h*.

tension in a way that would easily fit into many physiological schemes.

Drs L. Yu and R. N. St Onge supplied the spectra used to obtain the experimental numbers in Table 1. This work, using an electronic position sensitive detector, will appear elsewhere.

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Structural subunit in prisms of immature rat enamel

THE prisms of rat enamel, like those of other mammals, are composed of lath-like crystals with maximum widths of about 30 nm, arranged longitudinally in an organic matrix. The matrix is considered either to contain groups of fibrils or septae which orientate the crystallites, or to consist of an amorphous gel. The first view is based on the fact that pairs of electron-dense linear structures have been seen in fixed demineralised sections of immature enamel. These structures were about 5 nm wide and 15 nm apart, and parallel with the long axis of the prism. Furthermore, irregularly-arranged cross bridges have been described running between them^{1,2,3}. Physical techniques of structure determination, however, have failed to show evidence of periodicity in demineralised immature enamel⁴ and this has supported an alternative view that the matrix is an amorphous thixotropic gel⁵. This latter concept is consistent with the change in matrix composition when enamel matures⁶ and a simultaneous reduction in volume⁷. It does not, however, offer a satisfactory explanation of the orientation of the crystallites.

I have found another inconsistency—isolated prisms from immature enamel, which had been suspended in saturated calcium phosphate solution, could withstand ultrasonication for up to 1 h. This could not be attributed to an inter-lacing of the crystallites, because if 8 M urea solution

Fig. 1 Electron micrograph of demineralised crystallites isolated from prisms of immature rat enamel. Bar represents 100 nm. The arrow shows the junctional region between adjacent helices. The crystallites were obtained from albino rats (150 g). A 2-mm section of the soft enamel adjacent to the mature enamel was ultrasonicated for 10 min in 0.5 ml of distilled water which had been saturated with calcium phosphate. The crystallites were washed once with 1.5 ml of the suspending solution and deposited on copper grids with Formavar films. After drying, the grids were placed in a solution of 4% glutaraldehyde-0.1 M EDTA (pH 7.4) and withdrawn at 10-s intervals after 1 min. The grids were examined at $\times 7,500$ with an AEI EM6 B electron microscope to find suitable areas for photography.



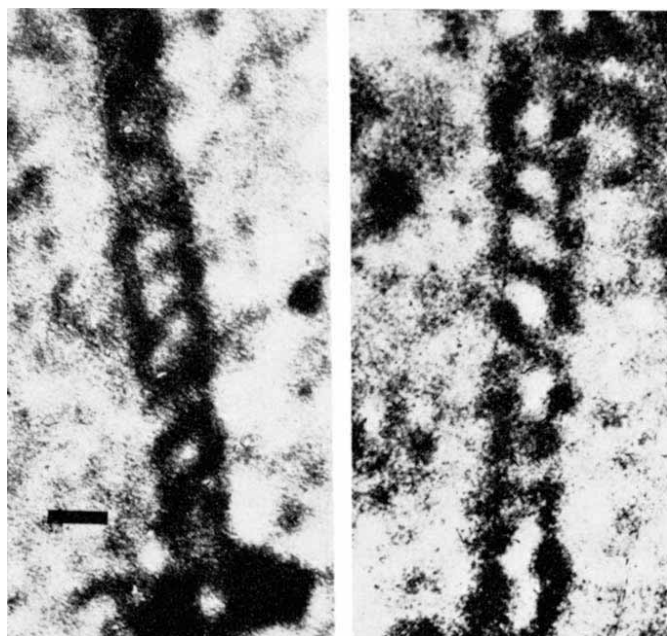


Fig. 2 Two selected areas of demineralised enamel crystallites showing helical arrangement. The bar represents 30 nm. Magnification in the electron microscope was $\times 7,500$ and subsequent enlargement was photographic to minimise beam damage to the unsupported specimens.

saturated with calcium phosphate was used to treat prisms either before or after ultrasonication they rapidly fell apart to yield individual crystallites. Urea (8 M) causes hydrogen bonds to break and this may have produced the effect on the prisms, either by allowing a structural network to separate, or by rendering a cementing substance more soluble. Sodium chloride solutions have been used widely to precipitate the soluble components of enamel matrix⁸, but a range of concentrations (0.3–2.0 M) failed to inhibit the action of 8 M urea solution, so it was concluded that a cement substance was not being made more soluble, and therefore that a structural framework was probably involved, part of the matrix being in that form.

To investigate this possibility further, isolated prisms which had partially unwound into separated crystallites were dried on to Formavar films on electron microscope grids, and demineralised carefully. As Fig. 1 shows, double lines of electron-dense material with cross bridges were observed, similar to those described in sectioned material. After examining many electron micrographs I concluded that this material consisted of double helices which seemed to have been wound around the crystallites. Selected areas which confirm this view are shown in Fig. 2. The helices varied in overall width from 15 to 30 nm and their pitch varied from 30 to 90 nm. The thickness of the coils of the helices was about 5 nm. Some appearances in Figs 1 and 3 suggested that bonding had existed where turns of helices on adjacent crystals came into contact. The variable pitch of helices would mean that they would not show periodicity and therefore would not be detected by physical techniques previously mentioned.

These observations and this interpretation could provide the basis for an explanation of the structural framework postulated here. This framework would consist of groups of helices whose long axes would be approximately parallel to one another and to the long axis of the prism. There would be multiple bonds between adjacent helices where their coils came in contact. Crystals threaded through the helices would brace the structure and give compressive strength. Against this view it could be argued that the structures described here were not present *in vivo*, but were aggregates of amorphous material which had been released

during preparation, and adsorbed on to the crystallites before dissolution. It is unlikely, however, that random forces could produce such consistent appearances. Furthermore, when the suspensions of rat enamel were made in 1.2 M phosphate buffer at pH 6.8, the helical structures could still be observed after demineralisation. As is well known, strong phosphate buffers desorb organic aggregates from hydroxyapatite^{9,10}, so that it is unlikely that aggregates could form in such a solution.

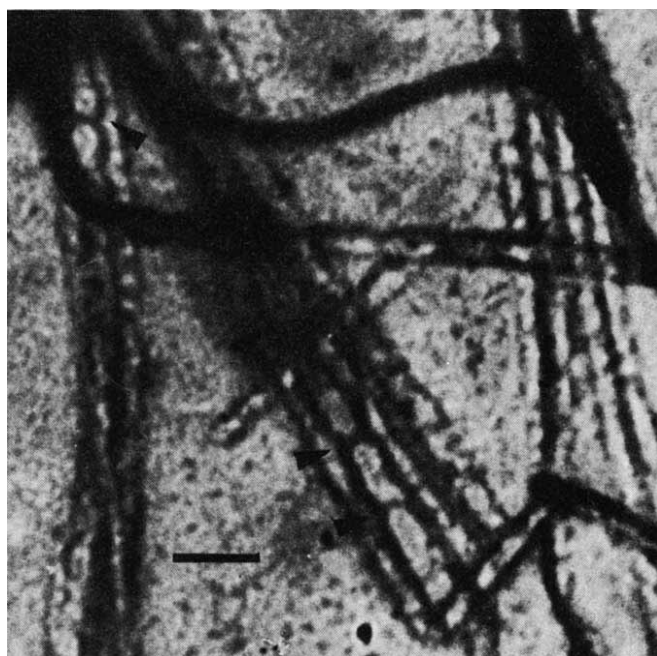


Fig. 3 Demineralised enamel crystallites showing evidence of bonding (arrowed) between helices on adjacent crystallites. The bar represents 100 nm.

It is interesting that structural helices with coils 5 nm thick have been described in F actin¹¹ (a double helix) and certain bacterial flagella (four or five helices)¹². The benefits and efficiency inherent in the use of helices as the basis of biological structures have been pointed out by Crane¹³ and Kushner¹⁴. It has also been suggested that some electron micrographs of the microtubules found in various animal and plant cells could be interpreted as showing helical arrangements of subunits, 5 nm in diameter¹⁴. It is possible therefore that the structures described here are the remnants of microtubules produced by the ameloblasts in the early stages of enamel formulation. Jessen¹⁵ has described groups of extracellular microtubules associated with the forming ends of rat ameloblasts. Furthermore, Jessen¹⁶ has demonstrated the presence of helical structures located in the mitochondria of the ameloblasts. They were of similar magnitude to those described above. The presence of such an unusual structure within the cells responsible for the formation of enamel provides strong support for the idea that the helices seen on enamel crystallites are an integral part of its structure at least at some stage in its development.

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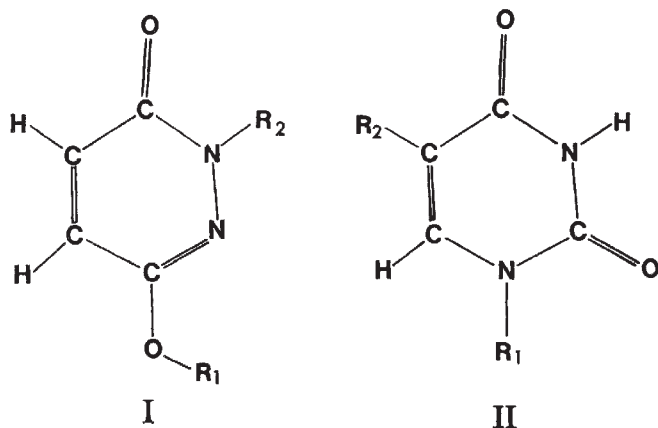
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Is maleic hydrazide a pyrimidine or purine analogue?

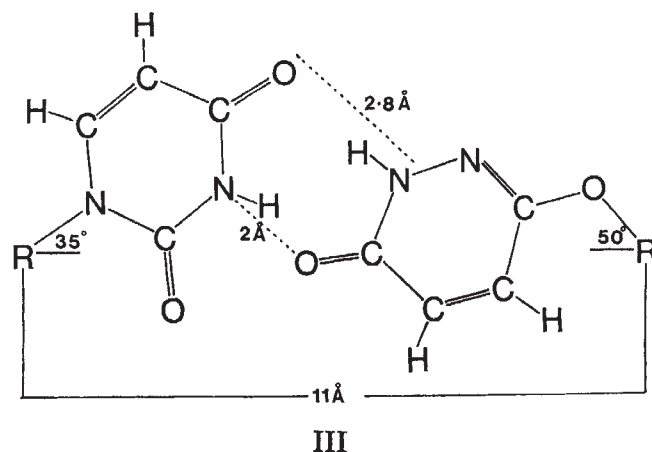
MALEIC hydrazide (structure I, $R_1=R_2=H$) is well known in agriculture as a growth inhibitor, but its mechanism of action is uncertain. Its close structural resemblance to the pyrimidine base uracil (structure II, $R_1=R_2=H$) has led to the suggestion that it replaces uracil in the RNA molecule^{1,2}, and although this theory is not universally accepted³, maleic hydrazide is known to be incorporated into RNA². The crystal structure of maleic hydrazide has now been elucidated (a full report will be published elsewhere) and a model of the molecular structure as found in the crystal has been used in attempts to form base pairs with the nucleic acid bases. The geometrical criteria of hydrogen bond formation suggests that maleic hydrazide can be regarded as either a pyrimidine or a purine analogue



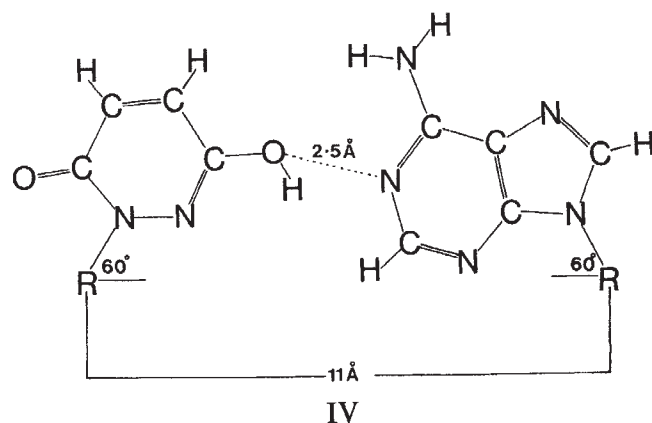
Maleic hydrazide crystallises in the space group $P\bar{1}$: $a=5.832$; $b=5.782$; $c=7.306$ Å; $\alpha=78.95^\circ$; $\beta=99.54^\circ$; $\gamma=107.23^\circ$; $Z=2$. Three dimensional intensity data were recorded on Weissenberg film packs using $CuK\alpha$ radiation and were measured photometrically. The structure was solved by application of the symbolic addition procedure⁴ and the rotation function⁵. Using three symbols, 81 reflections were phased, requiring the calculation of eight E maps, and one of these was found to be correct on the basis of orientation information provided by the rotation function. This structure was refined by the full matrix least squares procedure to a conventional R factor of 0.063, and corresponds to structure I.

The incorporation of a base or base analogue into the RNA structure requires the formation of a nucleoside, for example, uridine (structure II, $R_1=ribose$; $R_2=H$) resulting in a carbon–nitrogen bond between the base and ribose. Maleic hydrazide can form a bond either through its hydroxyl oxygen atom, giving the nucleoside analogue I ($R_1=ribose$, $R_2=H$) or through the NH group to form I ($R_1=H$; $R_2=ribose$). In the former case, base-pair formation with adenine is possible only if the $O-R \cdots R$ angle (shown as 50° in structure III) is increased to 100° . This angle is normally about 50° for Watson–Crick base pairs, and though distortions of about 20° are known⁶, an angle of 100° would require major distortion of the ribose–

phosphate chain. Base-pair formation is possible, however, with the pyrimidine bases uracil or thymine (structure II: $R_1=H$; $R_2=CH_3$)



Nucleoside formation through the nitrogen atom, on the other hand, could result in base-pair formation with adenine as shown on structure IV. Furthermore, with maleic hydrazide in the diketo form, a base pair of similar geometry is possible with guanine.



It seems, therefore, that if base pairing were the dominant mechanism for its action, maleic hydrazide should produce an inhibitory effect on the incorporation of any nucleoside into RNA. The application of a single nucleoside, however, should have only a very small effect on the incorporation of maleic hydrazide because of its nonspecificity.

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Corrigendum

In the article "Human breast cancer responsive to androgens in long term tissue culture" by M. E. Lippman, G. Bolan and K. Huff (*Nature*, **258**, 339; 1975), the authors omitted to thank the Michigan Cancer Foundation and Dr M. Rich for supplying them with a starter culture of their MCF 7 cell line.

matters arising

Lead levels in blood

THE article¹ by Waldron is misleading in its interpretation of the data selected, and in inadequate reference to the variables that were introduced during the period of the investigation, which in themselves precluded any reliable comparisons that might reasonably have been made between the series.

Waldron compared lead concentrations of capillary blood in the series one group of subjects, when it was accepted that the samples were suspect for reasons of contamination and haemo dilution², with venous blood samples from the same group of subjects in the second and third series, all of whose samples were analysed at the same laboratory. He did not, however, point out that 41 of these same individuals (18 males and 23 females) also gave capillary samples of blood in the second series. For valid comparisons to have been made between the series it would have seemed appropriate, in view of the transition from capillary to venous samples, to have included the matched capillary sample results of the series two group.

An assessment of the data in respect of the 41 subjects who provided capillary samples only in series one, capillary and venous samples in series two and venous samples only in series three is shown in Table 1. The mean values

nature of the series one capillary samples, his suggestion that the series two results support a rise in the blood lead concentrations cannot be properly upheld, as is shown by the results given in the Table.

Scrutiny of all of the main data showed no consistent differences in the three series of blood lead concentrations in the population in relation to residential distance from the motorway. Also, the ratio difference between the sexes remained virtually unaltered throughout the series. Had lead from the motorway been responsible for the increase in the blood levels observed, it would have been reasonable to have expected those living nearer to the motorway to have shown higher blood lead values than those living further away and difference in the ratio between the sexes to have diminished by virtue of absence of the majority of the male population at their various places of work during the day.

No control group was included in the study, a serious defect of the investigation, and no reference laboratory was used, which in view of the lack of agreement between the two laboratories engaged in the study, would have been of corrective value.

The lead in air findings of Butler *et al.*³ showed a low average concentration of 1 µg Pb per m³ air, as noted by Waldron. The apparent increases in

inconsistent with his notion that the aerosol is behaving as a gas.

The fact is that the lead in air findings of Butler *et al.* do not tie in with the increase of blood lead concentrations reported in the population under investigation. Because of inconsistencies and anomalies inherent in the investigation the results cannot be interpreted with any predictable level of confidence or be attributed to lead from traffic using the motorway.

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¹ Waldron, H. A., *Nature*, **253**, 345 (1975).

² Report of the Senior Administrative Medical Officer for Environmental Services to the Health Committee of the City of Birmingham, July 13, 1973.

³ Butler, J. D., Macmardo, S. D., Middleton, D. R., *Motor Vehicle Generated Pollution in Urban Areas*, Association of Public Health Inspectors Environmental Health Congress, Torbay, 1974.

WALDRON REPLIES—Barry's contention¹ that the blood lead levels in residents around Gravelly Hill have not been affected by the opening of the motorway link does not seem convincing. There is no *a priori* reason to suppose that one set of results is any more, or less, reliable than the other, and so the proper course should be to discover the mechanism whereby the blood concentrations have become elevated to their present levels. An investigation of the physical and chemical properties of the atmospheric lead particles seems at least one way in which this might be tackled.

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¹ Barry, P. S. I., *Nature*, **258**, 775 (1975).

Natural halocarbons in air and sea

THE amount of stratospheric chlorine released from natural CH₃Cl (ref. 1 and R. A. Rasmussen, personal communication), though certainly of scientific interest, is basically of slight relevance in consideration of the potential hazards to stratospheric ozone from chlorine atoms released from anthropogenic molecules such as CCl₂F₂ and CCl₃F (refs 2-4). In the first place, molecules such as CCl₃F have long atmospheric lifetimes

Table 1 Mean blood lead concentrations (µg Pb per 100 ml)

	No.	Series 1	Series 2		Series 3
		Capillary	Capillary	Venous	Venous
Males	18	18.1	17.6	16.8	25.6
Females	23	12.5	12.9	12.2	19.5

of the capillary blood lead results were comparable in series one and series two, but venous blood lead results showed an increase from series two to series three. In view of various anomalous aspects of the whole study these results require to be considered with circumspection.

Waldron's finding that capillary samples gave higher values than venous samples in a group of 96 individuals (from series two only) has been confirmed by other studies. Only 41 of these, however, could be directly compared with other series one results, so that, in conjunction with the suspect

blood lead levels at such low concentrations of lead in air are not supported by other published data. Neither can the suggestion that the pulmonary uptake of the lead aerosol from a motor vehicle exhaust may be considerably higher than 37 per cent be upheld in the light of the results of a recent study to be published shortly.

The apparent changes in blood lead levels force Waldron to include the possibility of 'the ingestion of lead contaminated dust'. Any particle size distribution, however, which would result in lead from motor vehicle exhausts being deposited in the dust must be

(estimated at 40–150 yr)³, and concern centres on the rapid increases in concentration observed for these compounds over the past several years. Natural CH_3Cl on the other hand, has a short lifetime (estimated at 0.37 yr) and should already be present at a steady level, at least on an annual average basis.

The lack of relevance of the natural content of stratospheric chlorine to these anthropogenic concerns, however, has an even more fundamental basis. All calculations to date indicate a progressive, roughly linear effect of added stratospheric chlorine on the depletion of the average ozone concentration, at least for changes of less than 10%. Consequently, the expected depletion in ozone from the continuing addition of anthropogenic chlorine is almost independent of whether the natural ozone level used as the baseline for comparison involves a 0%, 1%, or 5% component in its overall destruction from ClO_x of natural origin.

Ozone removal by stratospheric chemical processes predominantly involves the recombination of O_3 and O through NO_x catalysis, with lesser contributions from HO_x catalysis and direct reaction. Johnston (unpublished) has estimated that other processes could account for the additional, natural removal of as much as 15% of the ozone content, without affecting the consistency in present calculations of the balance between ozone formation and destruction; this additional destruction (15%) is the equivalent of about 6,000 parts per 10^{12} of stratospheric chlorine. Volatile organic halocarbon contributions of natural origin—chiefly, the recently discovered atmospheric CH_3Cl —total only about 1,000 parts per 10^{12} , while average stratospheric contributions from inorganic sources (sea salt, volcanoes) are much smaller than those from organic sources. The discovery of $\geq 5,000$ parts per 10^{12} of additional, natural, stratospheric chlorine now seems very unlikely.

These considerations of the anthropogenic against the natural ClO_x catalysis of ozone depletion parallel those made earlier for anthropogenic against natural NO_x catalysis. The detailed findings of the US Climatic Impact Assessment Program have shown that the addition of substantial quantities of anthropogenic NO_x would cause the extensive depletion of average ozone levels, despite the fact that these ozone levels are at present controlled chiefly by the reactions of natural NO_x .

Lovelock¹ has also argued that molecules such as CCl_2F_2 and CCl_3F are much more stable in the stratosphere than CH_3Cl . Present measurements and calculations suggest that is not the case. The tropospheric stability of CH_3Cl is certainly far less than that of CCl_2F_2 and CCl_3F because of the vulnerability of the former to attack by tropospheric OH radicals. In the stratosphere, however, with lower temperatures and lower OH

Matters arising

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concentrations, photolysis by solar ultraviolet radiation generally proceeds at a faster rate for CCl_3F (and at a comparable rate for CCl_2F_2) than the rate of attack of OH on CH_3Cl at the significant stratospheric altitudes.

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¹ Lovelock, J. E., *Nature*, **256**, 193 (1975).

² Molina, M. J., and Rowland, F. S., *Nature*, **249**, 810 (1974).

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⁴ Cicerone, R. J., Stedman, D. H., and Stolarski, R. S., *Geophys. Res. Lett.*, **2**, 219 (1975).

LOVELOCK REPLIES—Future generations will owe a debt to Molina and Rowland and to other scientists who have warned of the hazards to ozone by depletion by odd chlorine and odd nitrogen. I do not question their concern nor in broad outline their aeronomic models. There seems to be little doubt that in the long term the unrestrained release of fluorochlorocarbons to the atmosphere could have damaging and long lasting consequences.

Where I differ is on what constitutes a hazardous level of these compounds, and when it will be reached. At present the input of chlorine to the stratosphere from fluorochlorocarbons is small compared with that from CH_3Cl and CCl_4 . Furthermore, the atmospheric concentrations of the fluorochlorocarbons is no longer rising rapidly, possibly in part a consequence of the public recognition of the problem. The concentration of CCl_3F , for example, is still less than 10^{-10} per volume, perhaps one tenth that of CH_3Cl .

I concede that my remarks on the rapidity of the destruction of CH_3Cl in the stratosphere may be wrong. But if the mean global concentration is taken as 10^{-9} by volume then it would be responsible for the depletion of 1.6% of the stratospheric ozone. This is twice that of the combined effects of the two fluorochlorocarbons CCl_3F and CCl_2F_2 .

It is known that N_2O varies very substantially in concentration^{1,2} but measurements of CH_3Cl have only been made during the past 10 months. It is much too soon to conclude that its aerial concentration is constant. If 'slash and burn' agriculture proves to be a major source then CH_3Cl concentration may vary in proportion with this activity.

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¹ Goody, R. M., *Planet. Space Sci.*, **17**, 1319 (1969).

² Schutz, K., Junge, C., Beck, R., and Albrecht, B., *J. geophys. Res.*, **75**, 2230 (1970).

Latent tumour metastases

THE report by Eccles and Alexander¹ illustrates in an experimental system a phenomenon which is a familiar one with human tumours, which I have referred to as occult residual cancer^{2,3}. Sometimes the sudden appearance of frank metastases years after the apparent complete local removal of the primary tumour can be related to some particular event, as for example the sudden appearance of metastatic melanoma in a patient who subsequently developed a carcinoma of the breast and was treated by mastectomy and X-ray therapy⁴. A remarkable feature in this patient was that initially the melanoma metastases were confined to the area irradiated, though they subsequently became generalised. In other patients no cause can be discerned. This is exemplified by a patient⁵ who received a renal allograft after developing carcinoma first in one and then in the other of his own kidneys. When the second kidney was removed the tumour was found to be invading the renal vein and the prognosis was thought to be very poor. In spite of immunosuppression—or perhaps because of it for the azathioprine he received may have had an antitumour effect—he remained apparently tumour free for almost 7 yr, and then suddenly developed metastatic renal carcinoma first in his lungs and then elsewhere.

The clinical importance of this phenomenon is clear, but it has proved difficult to investigate because of the lack of a suitable experimental model. Fortunately Eccles and Alexander have now made good this deficiency.

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¹ Eccles, S. A., and Alexander, P., *Nature*, **257**, 52–53 (1975).

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reviews

THE 500th anniversary of Copernicus' birth was marked by numerous conferences, more as gestures of commemoration than scholarship. Although the Washington meeting of December 27-28, 1972 was no exception, some of its papers are nevertheless interesting, and their publication* is accompanied by many splendid photographs of early astronomical books and instruments.

An excellent beginning is John North's "The Medieval Background to Copernicus," an examination of the relation of Copernicus' work to the mathematical astronomy of Ptolemy and the later Middle Ages and to the natural philosophy of Aristotle and the scholastics. North emphasises the close relation to Ptolemy and Aristotle, and with good reason minimises the influence of scholastic speculations on the rotation of the earth. A following paper by Jerzy Dobrzycki on Copernicus' observations takes up, among other things, the idealised precision of the observations in *De revolutionibus*, largely serving the didactic purpose of demonstrating the methods of deriving parameters.

Later in the volume comes a well-illustrated paper by Owen Gingerich on the importance of printing to the development of the exact sciences in the Renaissance and on Copernicus' personal library of printed books. Stillman Drake nicely contrasts Giordano Bruno's eccentric and un-Copernican theory of the infinity of the universe with Kepler's painstaking mathematical development of Copernican theory and Galileo's independent investigations of mechanics that he only later turned to the defence of the motion of the earth. Although Drake acknowledges Kepler's (ultimately incorrect) physical theorising, its really great importance for Kepler himself needs further emphasis and investigation. Nevertheless, the paper is filled with shrewd and learned observations. Alexander A. Mikhailov's

"On the Quest for Direct Proofs of the Earth's Motion" is concerned primarily with the 300-year search for stellar parallax, in the course of which the aberration of light and double stars were unexpectedly discovered.

A session of five papers under the heading "The Nature of Scientific Revolutions," is concerned partially

tion of whether a crisis in astronomy precipitated the work of Copernicus, and concludes in the negative. He analyses error patterns in ephemerides of the sixteenth century, a very interesting subject on which he has written in the past, and shows that they are much the same before and after Copernican parameters were

incorporated into ephemeris computation. Much of the paper, however, is spent arguing against the notion that Ptolemaic theory had become encumbered with additional complications before the time of Copernicus.

Dudley Shapere considers in what sense the work of Copernicus constitutes a scientific revolution. He concludes that what was revolutionary was the degree of unification of the planetary system achieved by Copernicus' explanation of the second anomaly, bringing with it the fixed order and distances of the planets (benefits that, he astutely remarks, should not be referred to as "aesthetic"), and the persuasiveness of this unification for considering the theory real rather than fictional.

Thomas B. Settle examines as a specimen of change of theory or paradigm Galileo's two descriptions of the motion of a falling body, the first, from the *De motu* of 1590, that after an initial

acceleration the body reaches a uniform velocity, and the second, formulated by 1604 but not published until the *Discorsi* of 1638, that the distances are as the squares of the time intervals. Can this change, he asks, be described as a transition from normal science, through a crisis, to the formulation of a new paradigm—in other words, as a miniature scientific revolution with a discontinuity? No, he answers, because in further work on *De motu* Galileo devised a description of the initial acceleration as a (hypothetical) series of velocity increments per unit distance convergent on the uniform velocity, and this was a step toward

Copernican revolution



N. M. Swerdlow

with Thomas S. Kuhn's *The Structure of Scientific Revolutions* and its application to the Copernican Revolution. The first, by Richard Berendzen, compares the work of Copernicus with the early twentieth century discoveries concerning spiral galaxies and the extragalactic structure of the universe. Berendzen has done a good deal of work on the latter, but this paper seems an unfortunate effort at an uninformative comparison. The treatment of Copernicus is merely cliché; although many secondary sources, good and bad, are cited and quoted, few have been carefully read.

Owen Gingerich addresses the ques-

Fungal poisons

Molds, Mushrooms and Mycotoxins. By Clyde M. Christensen. Pp. 264. (University of Minnesota: Minneapolis, May 1975.) \$11.50.

THIS is the latest in a series of books on fungi by the author. Four chapters deal with the toxic effects of fungi on man and animals. *Amanita*, *Claviceps*, *Penicillium*, *Aspergillus* and *Fusarium* species are considered together with a variety of other fungi. Two overlapping categories are recognised: those fungi which are directly toxic as in the case of *Amanita* (amanitins and phalloidin), *Claviceps* (ergot alkaloids) and *Aspergillus flavus* (aflatoxin), and those which produce toxins in plant material either in the field or during storage (mycotoxins—for example, as from *Aspergillus* spp.)

As in other books describing the effects of toxins, the accounts are arresting, even lurid. It is surprising how much 'poison' can be ingested in a typical western diet; certainly, black pepper and peanut addicts might note the dangers arising from aflatoxin contamination. It might have been stressed more, however, that the presence of the organism or its spores does not necessarily indicate presence of the toxin. The pharmacology of the toxins is only briefly discussed, perhaps

because, in general, comparatively little has been done.

Other chapters deal with dissemination and the allergies arising from spores released by fungi, fungi pathogenic in animals (including man), such as those producing ringworm, and fungi which attack 'living' wood and timber; the near impossibility of protecting susceptible living wood from attack is disturbing. The last chapter on evolution is barely relevant but helps to explode the myth that fungi arose from algae.

The text is highly readable; by turns humorous, facetious and, occasionally, prejudiced. It is pleasant to know that 'nematodes do not have the brains to be bored'; also that locusts almost entirely consumed by *Massospora* can still 'laugh and chat'—enviable sang-froid! Documentation is adequate (102 references are cited) but the 'popular' nature of the text results in debatable generalisations and some errors of fact and judgement; for example, catching a nematode on a sticky, hyphal peg is not mechanically comparable to catching a snake with adhesive on the end of a pencil eraser.

The book is suitable for the lay reader as virtually all chemistry is omitted and scientific terms are explained in detail. It can be used as a useful reference by the professional mycologist and to stimulate

students' interest to encourage them to do much-needed research in the field of mycotoxicology. Farmers and agriculturalists might benefit from the subjects covered; numerous examples throughout the book indicate that more knowledge here might be of considerable value in offsetting hazards arising from fungal activity.

A. G. Dickerson

Dynamic processes

Dynamic Nuclear Magnetic Resonance Spectroscopy. Edited by Lloyd M. Jackman and F. A. Cotton. Pp. xiv+660. (Academic: New York and London, May 1975.) \$48.00; £23.05.

ALMOST since its first observation in bulk matter nuclear magnetic resonance (NMR) has been used for the study of molecular motion in solids and liquids. It was not until 1953, however, that Gutowsky and Saika suggested that it might be used to study chemical exchange dynamics in the liquid state. It is with those dynamic processes, chemical exchange, rotation about chemical bonds, molecular rearrangements and changes of conformation, that this book is concerned.

The first 160 pages are devoted to the theory of the effects of chemical exchange on NMR line shapes and widths and the nuclear relaxation times. The subject is most thoroughly covered, beginning with a delightfully clear historical introduction by H. S. Gutowsky, followed by four more chapters, some of them rather heavy going, by a distinguished group of experts. The fifth chapter, by R. Freeman and H. D. W. Hill is an elegant exposition of the problems of measuring spin-spin relaxation times in high resolution spectra, a procedure of such difficulty that it has not previously been much used.

The remaining 500 pages of the book, comprising ten further chapters, are concerned with an exhaustive review of the quite remarkable range of molecular processes which have been studied using this method, most of them involving intramolecular rearrangements in one form or another. No-one could fail to be impressed by the variety of systems studied, or by the ingenuity of the investigators, deriving information mostly unobtainable in any other way. The last chapter, by E. Grunwald and E. V. Ralph, on proton transfer, is the only one specifically concerned with intermolecular exchange.

This formidable and authoritative book is essential reading for organic and inorganic chemists concerned with molecular rearrangements; though its price may deter private buyers, it is nevertheless good value in these days of high printing costs. R. E. Richards

making distances per unit time proportional to the odd number series.

In the last paper, "Einstein on Scientific Revolutions," by Martin J. Klein, we find that Einstein wished to restrict the term to transformations, in Klein's words, "on the scale of the French or Russian Revolutions." His own series of papers on thermodynamics and statistical mechanics leading up to the Brownian motion paper of 1905—generally considered the definitive proof of atomic theory and the statistical nature of the second law—Einstein did not consider revolutionary because he was simply working through the consequences of mechanics. Nor did he consider the light quantum or special relativity theories of 1905 to be revolutionary, but only of heuristic value, as he described them both. Why? What Einstein did consider revolutionary was Maxwell's theory. But electromagnetic field theory was one thing and mechanics another. Reconcile

them, that is, unify the foundations of physics, and that, Einstein believed, would be a Scientific Revolution. Indeed it would. This paper is a gem, and should be required reading for anyone given to evaluating scientific discoveries. These five papers are followed by a lengthy discussion that is partly interesting and partly rambling.

Finally, there are papers concerned more with cultural or intellectual history. Benjamin Nelson compares the reception of Copernicanism with regard to "probability" and "reality" in Europe and to the Jesuits in China. Robert Palter's "Some Episodes in the History of Copernicanism" contains some observations on later opinions of Copernicus, but what this curious, wandering essay is about eludes me. Edward Rosen contributes a diatribe against an unnamed writer (Arthur Koestler), whom he calls "our detractor," that violates every canon of scholarship and taste. Koestler's *The Sleepwalkers*, even if it contains errors, is an intelligent study that does not deserve this rude treatment by a professional historian of science. "The Humanistic Significance of Our Copernican Heritage" by Jerome R. Ravetz is a model of eloquence. Learned, stimulating, and philosophical by turns, Ravetz considers Copernicus' life and work, his followers, interpreters, and critics, and our Copernican heritage.

**Copernicus: Yesterday and Today.* (Proceedings of the commemorative conference held in Washington in honour of Nicolaus Copernicus, Vistas in Astronomy, Volume 17.) Edited by Arthur Beer and K. Aa. Strand. Pp. xxxiii+225. (Permagon: Oxford and New York, 1975.) Subscribers' price: \$40.00, £17.00; Non-subscribers: \$50.00, £21.00.

Variability of mammals

Variability of Mammals. By A. V. Yablokov. Translated from Russian. Pp. xv+350. (Amerind: New Delhi.) \$12.50.

THIS book deals with an increasingly felt need for a unified approach to the collation of data on population morphology. In the past this has been in the province of geneticists, comparative anatomists, zoogeographers and taxonomists, but now data are collected and required by a far wider spectrum of biologists. In synthesising this review, Professor Yablokov has drawn heavily on data collected by Russian biologists which are not readily available in the west. Useful as that approach is, it inevitably means that field data must be accepted at face value for accuracy of measurement and no allowance made for error; the book does not caution the reader to be on his guard to interpret the facts accordingly. Nevertheless, a great deal of work has yet to be done before a general theory of variability can be advanced, and one must beware of throwing the baby out with the bath-water.

The first part of the book lays the foundations for a discussion of the adaptiveness of variability by using a coefficient of variation of a series of traits as an index of the different ecological conditions encountered by different populations of the same species. Since few distribution curves for given traits correspond to a normal distribution, the best means for expressing differences is a problem yet to be solved by morphologists. The dimensionless coefficient (the standard deviation divided by the sample mean and expressed as a percentage) is used throughout the book to compare widely different traits between populations within a species, within a genus, between closely related genera, between subfamilies and within an order. The result of the author's examination of traits in the Pinnipedia indicates that the coefficient of variation between two populations may be significantly greater than the coefficient of variation for populations from two different subfamilies. Professor Yablokov argues for a rationalisation of the classification of variability in various independent ways such that the processes of evolution of different populations can be directly compared. This is particularly valuable when comparing the variability of natural and experimentally controlled populations and that between populations experiencing clearly distinguished and different environmental conditions.

A heavy, laboured style makes for difficult reading, but this may be a result of the translation. Several line diagrams have insufficient legends and the halftone plates are so badly reproduced as to be almost without value. Nevertheless, the book is a useful starting point for the serious student of population evolution and mammalian—in particular pinnipede—biology, and it should fulfil a role in describing the baseline of this important area of biology.

D. Michael Stoddart



Johannes de Ketham's *Fasciculus medicinae* shows the first printed illustration of a human dissection, attributed by some scholars to the artist Gentile Bellini. Originally published in 1491 in Venice shortly before Copernicus became a medical student in nearby Padua. (Yale Historical Medical Library.) *The Nature of Scientific Discovery.* (A symposium commemorating the 500th anniversary of the birth of Nicholas Copernicus.) Edited by Owen Gingerich. Pp. 616. (Smithsonian Institution Press: Washington, DC, June 1975.) \$15.00.

Energetic perspective

Perspectives on Energy: Issues, Ideas and Environmental Dilemmas. By Lon C. Reudisili and Morris W. Firebaugh. Pp. xii+527. Oxford University: London, New York and Toronto, August 1975. Boards £6.85; paper £4.00.

THE objective of the book is to provide clear and concise information on the arguments in America for assuring energy supplies, while not conflicting too much with environmentalists who oppose some of the methods. To pro-

duce what the authors believe to be a balanced and representative analysis in areas of controversy, they have made a selection of articles from the recent literature illustrating the energy and environmental dilemmas—reviewing the prospects of fossil and nuclear fuels as energy sources, considering alternative energy possibilities and finally ideas on energy policy for the future.

In each of five chapters the layout is similar—unusual but interesting. Each begins a group of photographs (generally clear and relevant to the subject matter of the papers in the chapter) and each is introduced by a few pages of synopses or commentary.

Glenn T. Seaborg, ex-US Atomic Energy Commission but now in his haven at Berkeley, California, repeatedly refers in his foreword to the “readings” in the book. And so the individual papers are, many of them original gems of authority. The book can be picked up and opened almost at random to find a self-sufficient specialist view on some subject of relevance in today's energy world. The 527-page tome, therefore, is really a short daily ration of “sifting and winnowing” to reach an intelligent informed opinion on energy politics assisted by the experts.

Chauncey Starr sets the pace with the “Realities of the Energy Crisis”, and M. King Hubbert continues with a “Survey of World Energy Resources”. The “Case for Nuclear Energy” is given by B. I. Spinrad followed by half a dozen pages nibbling away at topics like “Reactor Safety”, “Disposal of Nuclear Wastes”, and “Dilemma on Fission Energy and Pugwash warnings”.

The book is rich in clear diagrams and useful tables, indeed worth at least the paperback price for the 24 in the Hubbert paper alone—more than one per page. There are plenty of references given in most of the 40 papers, and, as if that is not enough to keep one going into the small hours there is a suggested reading list at the ends of chapters.

Seaborg opens the debate with worry about a resource crisis (certain metals, food, water, energy) and wonders whether there is sufficient concern for the future. Ruedisili and Firebaugh provide plenty of thought-provoking material and cleverly end the book with Seaborg's answer to his own question on ideas for a “Recycle Society”. Scrap would become secondary material but new material would be used rarely and only for top-up of the stock in use. Design for non-obsolescence, communications instead of transport, avoidance of urban sprawl and improvement of the energy situation could all contribute to a better life twenty years from now; that's not far away, he warns, and we had better get moving.

G. R. Bainbridge

Clarifying nuclear structure

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Aage Bohr (left) and his collaborator Ben Mottelson at work

Nuclear Structure. Volume 2: Nuclear Deformations. By Aage Bohr and Ben R. Mottelson. Pp. xiii+748. (Benjamin: Reading, Massachusetts, November 1975.) \$37.50.

For more than twenty years Bohr and Mottelson have devoted themselves to studies of nuclear structure, and have established in Copenhagen one of the world's leading centres for theoretical physics. Together with Professor James Rainwater, they have recently been awarded the 1975 Nobel Prize for physics in recognition of this work. Their seminars and lectures have gradually developed into a massive treatise of three volumes, of which this is the second. The first volume, on nuclear single particle motion, was published in 1969, and the third, on nucleonic correlations, is in preparation.

The central theme is the interplay of the independent particle and the collective aspects of nuclear structure, and the way this shows itself in the vibrational and rotational spectra of nuclei. The first volume covered the independent particle aspects in detail, and this volume is devoted to nuclear deformations, with chapters on rotational spectra, one-particle motion in non-spherical nuclei and on vibrational spectra.

The first of these discusses in turn the symmetry operations for rotating nuclei, the energy spectra and intensity relations for axially symmetric nuclei, the coupling between rotational and intrinsic motion for axially symmetric nuclei, and rotational spectra for systems without axial symmetry, and concludes with an appendix on the particle-rotor model.

The next chapter brings out the connection with the single-particle motions of the first volume by showing what happens to the motion when the potential is deformed. Successive sections are devoted to the stationary states and wave functions of particle motion in a spheroidal potential, and the classification of odd-A spectra and moments and transitions. This is followed by an appendix on scattering by non-spherical systems.

Quantum theory

Introduction to Axiomatic Quantum Field Theory. (Mathematical Physics Monograph Series, Volume 18.) By N. N. Bogolubov, A. A. Logunov and I. T. Todorov. Pp. xxvi+708. (Benjamin: Reading, Massachusetts, and London, July 1975.) Cloth \$32.50.

THE publishers of this book are too optimistic when they say "it requires little preliminary knowledge of either mathematics or quantum field theory", and I prefer the authors' own assessment that "it would be useful to have an elementary knowledge of quantum field theory" and some undergraduate mathematics. It is a more complete treatise than *General Theory of Quantised Fields* by R. Jost (*Am. math. Soc.*, 1965) or *PCT, Spin and Statistics, and All That* by R. F. Streater and A. S. Wightman (Benjamin, 1964), and is naturally more up to date. It follows the same general lines, but with more detail in places and some topics are taken further. The inclusion of more group theory, wave equations and second

The last chapter, occupying over half the book, is devoted to vibrational spectra. There is a full account of the quantal theory of harmonic vibrations, the normal modes of vibration and the sum rules for multipole oscillator strength. Sections follow on particle-vibration coupling and anharmonicity in vibrational motion; there are also appendices on the liquid drop model of vibrations and rotations and on the five-dimensional quadrupole oscillator.

The level of presentation is such that it can be understood by a graduate student, and yet it is so comprehensive that most aspects of nuclear collective motion are covered. In a book of this kind there is a danger that the reader is overwhelmed by a mass of detail, and fails to see the essential theoretical structure underlying the specialised applications. Bohr and Mottelson attempt to avoid this by dividing the book into three levels: text, illustrative examples and appendices. The text contains the basic theory, presented in as straightforward and uncluttered a manner as possible, and its application to analyse the structure of particular nuclei is relegated to the second level. The appendices contain standard mathematical derivations and formulae. On the whole this division is successful in clarifying the presentation.

As would be expected, the treatment is thorough and detailed throughout, so that it will remain an indispensable work of reference for many years to come.

P. E. Hodgson

quantisation certainly fills a gap in the earlier books. Scattering theory is better covered, and provides a logical introduction to the S-functional approach of Bogoliubov and to the perturbation expansion of Feynman and Dyson. This section ends with a brief account of renormalisation theory. There follows an account of the general results of the theory, usually in great detail but occasionally avoiding overlap with the other books by referring to them. Infinite-component fields and parastatistics are among new topics. The book ends with a very brief account of the C*-algebra approach, and the progress of constructive field theory up to 1971.

The bibliography is quite large and contains very useful references to Russian work on this subject. The book, being typed and photocopied, is rather bulky. It is well translated, authoritative and suitable as a companion and guide for postgraduate students in physics or mathematics studying relativistic quantum mechanics, and for other research workers seeking a way in to this difficult subject.

R. F. Streater

Photo: AP

Physics and Chemistry of Surfaces. By Jacques Oudar. Pp. xii+130. (Blackie: Glasgow and London, July 1975.) £6.55.

PROFESSOR Oudar's book aims to provide an introduction to two aspects of surface chemistry. The first section is devoted solely to chemisorption of gases on metals, the rest of the book concerns the oxidation of metals. Clearly with less than 120 pages of script and that divided into sixteen chapters, no single topic can be discussed in any detail. Thus the book provides a cursory view of a field which has undergone major development in the last decade. The strength of the book is that it has set its descriptions of modern techniques and recent results in the context of the older approaches to the subject.

As a textbook it is too short to be of value as a source of reference or an extension of lecture notes. It is, however, easy to read and very well illustrated. There are occasional errors in the transcription and some of the concepts presented are not universally held. It does provide, however, a valuable overview of the subject but it is rather expensive for its length.

A. E. Lee

The Early Development of Mammals. (The Second Symposium of the British Society for Developmental Biology.) Edited by M. Balls and A. E. Wild. Pp. vii+410. (Cambridge University Press: Cambridge and London, 1975.) £18.00; \$49.50.

THIS is an excellent book, covering many of the current lines of research in mammalian embryology. The 23 chapters include culture and storage methods, fertilisation, parthenogenesis, differentiation, expression of genes and antigens, induction, development of the lymphoid and haemopoietic systems, teratomas and teratocarcinomas, sex reversal, athymia and cell death. Inevitably, the mouse holds the centre of the stage, but other mammals make brief appearances from time to time.

The 30 contributors are all actively engaged in research in mammalian development and write with authority. Most give a short review of previous work in their particular field followed by an account of recent experiments. In general, the standard of presentation is high. The style is concise but readable, the illustrations and index are adequate and there is the minimum of repetition between chapters. An occasional paragraph is muddled

or incomprehensible and would have benefited from tougher editing, but such blemishes are few and become insignificant when set beside all that is good. The British Society for Developmental Biology, and particularly the two editors, have clearly had a major task in bringing together so much in one book and they are to be congratulated on the result.

The book will be of great value to all research workers on mammalian development and to many other biologists who want to know where the subject has got to. Its wide coverage and attractive presentation should also make it stimulating reading for teachers and students of undergraduate courses in developmental biology. Unfortunately, many potential readers will be put off by the price from buying the book for themselves.

D. A. T. New

Books brief

Chemistry of Viruses. Second Edition. (Springer Study Edition.) By C. A. Knight. Pp. x+325. (Springer: Berlin, Wien and New York, 1975.) DM48; \$20.70.

Knight has set out to produce a compendium of chemical information covering the major classes of viruses. The result is an impressive collection of data dealing almost exclusively with the virus particle and presented in a style somewhere between a handbook and a textbook. Purification and especially composition are considered in great depth, often including precise experimental details, and clearly reflect the author's wide experience and knowledge of the subject. Particularly useful is the treatment of the four virion components (protein, nucleic acid, lipid and carbohydrate) in terms of methods of purification, methods of analysis and function. Unfortunately there is no mention of the agarose gel electrophoresis and restriction endonuclease cleavage techniques which are currently much in use for nucleic acid analysis. Also absent is any reference to the cohesive, repetitious and inverted repetitious base sequences present at the ends of many types of viral DNA genome. The interactions between virus particles and chemical agents which lead to inactivation and mutation are

discussed in a clear and reasonably comprehensive manner albeit in somewhat less depth than earlier chapters. The final chapter collects together a set of topics which make curious bedfellows, for example, virus reproduction, genetic recombination, viroids, reconstitution, cell-free synthesis and origin of viruses. They are discussed very superficially, much like an afterthought, and could well have been omitted or included in a general introduction. But this is otherwise a minor criticism of a worthwhile book which will serve as a very useful source of information on most aspects of the virion.

D. A. Ritchie

Equilibrium and Non-equilibrium Statistical Mechanics. By Radu Balescu. Pp. xiv+742. (Wiley-Interscience: New York and London, 1975.) £16.20.

STATISTICAL mechanics is a fascinating and confusing subject: it is tremendously successful in practice, but its ultimate justification has proved surprisingly difficult to establish. And although there has been much recent work on foundations, it is forbiddingly difficult for the average physicist to understand. This book is the one to read in order to remove one's misconceptions and in order to achieve an understanding of the subject.

The style and presentation are admirably clear, and there are many enjoyable asides and perceptive comments. Among the applications selected for discussion, plasmas and classical gases predominate, and there is an excellent account of modern work on critical phenomena, including Wilson's theory. In the non-equilibrium section the emphasis is on kinetic equations and their justification by the methods of the 'Brussels school' of which the author is a distinguished member. There is a delightful appendix giving a nontechnical summary of recent work on the ergodic problem.

Obviously, in such a vast subject every author has his own preferences, and for my own taste there is not enough emphasis on specific quantum phenomena: quantum theory tends to be treated as a correction to classical physics, and some of the most exciting applications of statistical mechanics, to photons and superfluids, are barely mentioned. But within its self-imposed limits this book is certainly a very valuable addition to the literature and deserves to be widely studied.

E. H. Sondheim

obituary

Dr J. F. Heremans, Professor of Internal Medicine at the Catholic University of Louvain, died on October 29, aged 48. He received his medical degree with highest distinction, at Louvain in 1952, and after specialising in internal medicine, worked with Professor Waldenström in Malmö (1957–1958), and with Professor Kunkel at the Rockefeller Institute (1959). He became Professor at Louvain in 1965, and was one of the founder members of the newly created International Institute for Cellular and Molecular Pathology in Brussels. His expertise was recognised by the award of several national scientific prizes, and he was a member of many learned societies throughout the world, notably the European Society for Clinical Investigation, of which he was elected President in 1971.

While interested in all aspects of experimental medicine, his major re-

search interest was immunoglobulins. The most significant of his discoveries is this field was the isolation and characterisation of IgA.

Having established its chemical similarity to the other two major types of antibody molecule, he proposed the term 'immunoglobulin' as descriptive of all three, and afterwards played a large part in the development of our present unified concept of antibody structure and function.

Professor Heremans was always aware of the need for accurate quantitation of proteins, whether in the research or clinical fields, and it was under his direction that the Mancini Radial Immunodiffusion Technique was developed. More recently, a variety of automated immunoassay techniques suitable for such diverse applications as plasma protein screening and drug and hormone measurement have originated in his laboratory.

His encyclopaedic knowledge of protein structure and function is best illustrated by the compendious 'Molecular Biology of Human Proteins', written before his 40th birthday, in collaboration with Professor H. E. Schultze. Now a standard reference work, it also demonstrates his extraordinary linguistic ability; he was completely fluent in five European languages, and conversant with several others.

Professor Heremans was a man of very wide-ranging interests; he was a pianist of considerable talent and a keen amateur lepidopterist. He maintained a lively interest in the work of these in his laboratories, to whom he was always available to give advice. He leaves a wife and five children, by whom, as well as by his scientific colleagues and friends, he will be greatly missed.

John Peters-Dickie

announcements

International meetings

January 8–9, Molecular developmental biology, Glasgow (Department of Zoology, University of Glasgow, Scotland).

February 18, Materials microanalysis with less than 1,000 Å spatial resolution, Churchill College, Cambridge (The meetings secretary, The Institution of Metallurgists, Northway House, Whetstone, London N20 9LW, UK).

February 20, Great women in science, Boston (American Association for the Advancement of Science, 1176 Massachusetts Avenue, NW, Washington DC, 20036, USA).

February 25, Professional metallurgists in Europe, Imperial College, London (The meetings secretary, The Institution of Metallurgists, Northway House, Whetstone, London N20 9LW, UK).

Reports and publications

Other Countries

United States Department of the Interior: Geological Survey. Water-Supply Paper 2133: Surface Water Supply of the United States, 1966–70. Part 12: Pacific Slope Basins in Washington. Vol. 2: Upper Columbia River Basin. Pp. viii+674. Water-Supply Paper 2171: Ground-Water Levels in the United States, 1969–73. Southeastern States. Pp. v+250. (Washington, DC: Government Printing Office, 1975.) [311]

United States Department of Agriculture. Index-Catalogue of Medical and Veterinary Zoology. Supple-

ment 19, Part 5: Parasite-Subject Catalogue—Arthropoda and Miscellaneous Phyla. By Jane D. Rayburn, Martha Walker Hood, Margie D. Kirby and Judith H. Shaw. Pp. iv+403. (Washington, DC: US Government Printing Office, 1975.) \$4.60. [411]

A Selected and Partially Annotated Bibliography of Society, Ethics and Life Sciences. Compiled by Sharon Solliito and Robert M. Veatch. Revised by Diane Fenner. Pp. 56. (Hastings-on-Hudson: Institute of Society, Ethics and the Life Sciences, 1975.) [411]

United States Department of the Interior: Geological Survey. Bulletin 1211-F: Geologic Reconnaissance of a Proposed Powersite on the Maksoutof River near Sitka, Southeastern Alaska. By Alexander A. Wanek. Pp. iii+22+1 plate. Water-Supply Paper 2142: Quality of Surface Waters of the United States, 1969. Part 2: South Atlantic Slope and Eastern Gulf of Mexico Basins. Pp. x+625. \$4.35. Water-Supply Paper 2146: Quality of Surface Waters of the United States, 1969. Part 7: Lower Mississippi River Basin. Pp. xi+540. \$3.85. Professional Paper 813-C: Summary Appraisals of the Nation's Ground-Water Resources—Upper Colorado Region. By Don Price and Ted Arnow. Pp. vi+40+2 plates. \$3.15. Professional Paper 908: Simulated Effects of Oil-Shale Development on the Hydrology of Piceance Basin, Colorado. By John B. Weeks, George H. Leavesley, Frank A. Welder and George J. Saulnier, Jr. Pp. vii+84+1 plate. \$3.15. Professional Paper 930: A Hydrologic Assessment of the September 14, 1974, Flood in Eldorado Canyon, Nevada. By Patrick A. Glancy and Lynn Harmsen. Pp. vi+28. (Washington, DC: Government Printing Office, 1974 and 1975.) [411]

United States Department of the Interior: Geological Survey. Water-Supply Paper 2111: Surface Water Supply of the United States, 1966–70. Part 4: St. Lawrence River Basin. Vol. 1: Basins of Streams Tributary to Lakes Superior, Michigan and Huron. Pp. viii+754. \$5.10. Water-Supply Paper 2132: Surface Water Supply of the United States, 1966–70. Part 12: Pacific Slope Basins in Washington. Vol. 1: Pacific Slope Basins in Washington Except Columbia River Basin. Pp. viii+640. \$4.40. Water-Supply Paper 2148: Quality of Surface Waters of the United States, 1969. Parts 9 and 10: Colorado River Basin and The Great Basin. Pp. ix+348. \$2.65. (Washington, DC: Government Printing Office, 1974.) [611]

World Meteorological Organisation. Technical Notes, No. 136: Mulching Effects on Plant Climate and Yield. By J. W. Davies. Pp. v+92. No. 137: Meteorology and the Colorado Potato Beetle. By G. W. Hurst. Pp. iv+51. No. 138: Drought and Agriculture.

Report prepared by C. E. Hounam, J. J. Burgos, M. S. Kali, W. C. Palmer and J. Rodda. Pp. vi+127. No. 139: Climatological Aspects of the Composition and Pollution of the Atmosphere. By G. C. Holzworth. Pp. vii+43. No. 140: Upper-Air Sounding Studies. Vol. 1: Studies on Radiosonde Performance. By A. H. Hooper. Vol. 2: Manual Computation of Radiosonde. By R. E. Vockeroth. Pp. xii+160. No. 141: Utilisation of Aircraft Meteorological Reports. Prepared by S. Simplicio. Pp. iv+34. (General World Meteorological Organisation, 1975.) [611]

Australia: Commonwealth Scientific and Industrial Research Organization. Annual Report of the Minerals Research Laboratories, 1974/1975. Pp. 68. (Melbourne: CSIRO, 1975.) [611]

Australian Academy of Science. Report No. 19: A National System of Ecological Reserves in Australia. (Proceedings of a Symposium, Canberra, 31 October 1974.) Edited by Frank Fenner. Pp. 114. (Canberra: Australian Academy of Science, 1975.) [611]

CERN—European Organisation for Nuclear Research. CERN 75-12: A 256-Channel Pulse-Height Analyser. By J. C. Berset, G. Delavallade and J. Lindsay. Pp. v+45. (Geneva: CERN, 1975.) [611]

Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem. Heft 164: Der Gelbrost, Puccinia Striiformis West. IV. Epidemiologie-Bekämpfungsmassnahmen. Von Prof. Kurt Hassebraut und Prof. Gerhard Robbel. Pp. iv+183. Heft 165: 40. Deutsche Pflanzenschutz-Tagung in Oldenburg i.O., 6–10 Oktober 1975. Pp. 227. (Berlin-Dahlem: Biologischen Bundesanstalt für Land- und Forstwirtschaft, 1975.) [1011]

Smithsonian Contributions to Zoology, No. 185: Bredin-Archbold-Smithsonian Biological Survey of Dominica. The Family Dolichopodidae with Some Related Antillean and Panamanian Species (Diptera). By Harold Robinson. Pp. iii+141. (Washington, DC: Smithsonian Institution Press, 1975. For sale by US Government Printing Office.) [1011]

United States Department of the Interior: Geological Survey. Bulletin 1390: Monazite Placers in the Southeastern Atlantic States. By John B. Mertie, Jr. Pp. iv+41. 80 cents. Water-Supply Paper 1969: Water-Supply Development and Management Alternatives for Clinton, Eaton, and Ingham Counties Michigan. By K. E. Vanlier, W. W. Wood and J. O. Brunett. Pp. vii+111+3 plates. \$3.20. Professional Paper 437-H: Land Subsidence in the San Joaquin Valley, California, as of 1972. By J. F. Poland, B. E. Lofgren, R. L. Ireland and R. G. Pugh. Pp. v+78. (Washington, DC: Government Printing Office, 1973 and 1975.) [1011]